The Pathogenesis of GPCR-positive Breast Cancer

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Breast cancer is a heterogeneous disease with a variety of molecular drivers regulating its growth, survival and response to therapy. We have identified aberrant overexpression of the type I angiotensin II receptor (AGTR1), a G-protein-coupled receptor (GPCR), in a subset of luminal breast cancers (characterized as ER^+, PR^+, HER^-). Previously we found that Ang II, the ligand of AGTR1, stimulates the pro-inflammatory and pro-survival NF-κB transcription factor in vascular cells via activation of the CBM signalosome, a multi-protein complex consisting of three components: CARMA3, Bcl10 and MALT1. We have now demonstrated that the CBM signalosome similarly mediates NF-κB activation and promotes aggressive phenotypes in AGTR1-positive breast cancer.

Unexpectedly, overexpression of AGTR1 in the luminal ZR75-1 cell line (ZR75-AGTR1) caused epithelial-to-mesenchymal transition (EMT), identified by the conversion to mesenchymal morphology, downregulation of epithelial marker E-cadherin, and upregulation of mesenchymal markers N-cadherin, vimentin, as well as EMT transcription factors Snail and ZEB1. BT549, a breast cancer cell endogenously overexpressing AGTR1, also displays these mesenchymal characteristics. In BT549 cells, AGTR1 knockdown with siRNA and MALT1 knockdown with siRNA or shRNAs all reversed the expression of Snail. Moreover, disrupting MALT1 function in BT549 cells with siRNA or MALT1 protease inhibitors abrogated the migratory and invasive properties of these cells.

The protease-activated receptor 1 (PAR1) is another GPCR utilizing the CBM signalosome as downstream signaling mediator. We found that overexpression of PAR1 in MCF7 cell also resulted in EMT-like morphological changes, downregulation of E-cadherin, and upregulation of vimentin, Snail and ZEB1. In MDA-MB-231 cells, which endogenously overexpress PAR1, knocking down PAR1 with siRNA or knocking down MALT1 with siRNA or shRNAs led to reversal of Snail. Disrupting MALT1 function in MDA-MB-231 cells with siRNA or MALT1 protease inhibitors abrogated the cell migratory and invasive ability of these cells.

Importantly, gene set enrichment assay (GSEA) shows that MALT1 is strongly associated with EMT in human breast cancers and that EMT is one of the most dramatically induced pathways in AGTR1 or PAR1-positive triple-negative breast cancer. These results suggest that the CBM signalosome plays an important role in EMT in breast cancers harboring AGTR1 or PAR1 overexpression.

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Preface

Since I was a child, I was always interested in biology. The passing away of my father strengthened my resolve to unravel the convoluted mystery of disease. As time goes on, I have dedicated my life in studying the cause and development of diseases, hoping that one day people can be free of such affliction. Under the mentoring of Drs. Peter Lucas and Linda McAllister-Lucas at University of Pittsburgh, I have advanced closer to my dream. For me, obtaining the PhD degree was a long and winding road. The support and understanding from Peter and Linda gave me strength to overcome obstacles, finally winning the laureate of PhD. I appreciate the assistance from everyone in Lucas' laboratory. I have to thank Prasanna Ekambaram for his efforts and unselfish contribution on this project. I also want to thank Dong Hu for providing inspirational idea and technical support to my research. Moreover, I wish to thank Linda Klei, Vincent Concel, Jing Cheng, Kelly Bailey, Lisa Maurer, Tanner Freeman, Heejae Kang, Claire Julian, and Xiaoshuang Lyu. It was a great joy and honor working with them all.

In addition to our talented laboratory members, I was lucky enough to have a dissertation committee that dedicated themselves to helping me succeed in my graduate program. Drs. Marie DeFrances and Paul Monga from Department of Pathology, and Drs. Steffi Oesterreich and Romero Guillermo from Department of Pharmacology & Chemical Biology all guided me with their vast knowledge in cancer biology, constructive suggestions and great patience. For these, I am deeply grateful.

Finally, I have to give my special thanks to our program director, Dr. Wendy Mars, for supporting me, believing in me, from the very beginning to my final accomplishment in the graduate program. I thank you from the bottom of my heart.

This work is dedicated to my dear grandparents.

I owe you so much.

I did not let you down.

Jia-Ying Lee

April 2019

In loving memory of Ching-ho Lee and Mei-fong Lee-Hsu

1.0 Introduction – Part 1

Breast cancer is a heterogeneous disease with different molecular alterations driving its growth, survival and response to therapy. We recently identified a subpopulation of breast tumors that harbor overexpression of angiotensin II receptor type I (AGTR1), a G-protein-coupled receptor (GPCR). In this dissertation, we look into the influence of AGTR1 overexpression on the tumorigenesis of breast cancer.

1.1 Breast cancer

Breast cancer is the most commonly diagnosed and second leading cause of cancer-related deaths in women worldwide (Figure 1).[1] By evaluating the pattern of expression of the hormone receptors (HR) estrogen receptor and progesterone receptor, and HER2, breast cancer is classified into three major molecular subtypes: luminal tumors (HR+/HER-); HER2 amplified tumors; and tumors with lack of expression of the three receptors, generally referred to as triple-negative breast cancer (HR-/HER-).[2] Each of these subtypes shows various risk factors for therapeutic response, disease progression, and preferential organ sites of metastases.

Estimated Deaths

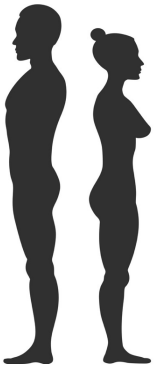
Males				Females		
Lung & bronchus	83,550	26%		Lung & bronchus	70,500	25%
Prostate	29,430	9%		Breast	40,920	14%
Colon & rectum	27,390	8%		Colon & rectum	23,240	8%
Pancreas	23,020	7%		Pancreas	21,310	7%
Liver & intrahepatic bile duct	20,540	6%		Ovary	14,070	5%
Leukemia	14,270	4%		Uterine corpus	11,350	4%
Esophagus	12,850	4%		Leukemia	10,100	4%
Urinary bladder	12,520	4%		Liver & intrahepatic bile duct	9,660	3%
Non-Hodgkin lymphoma	11,510	4%		Non-Hodgkin lymphoma	8,400	3%
Kidney & Renal pelvis	10,010	3%		Brain & other nervous system	7,340	3%
All sites	**323,630**	**100%**		**All sites**	**286,010**	**100%**

Figure 1 - Ten leading cancer types for the estimated deaths by sex, United States, 2018.

1.1.1 Risk factors of breast cancer

About one-third of postmenopausal breast cancers are thought to be associated with behaviorally modifiable factors, such as postmenopausal obesity, physical inactivity, use of combined estrogen and progestin menopausal hormones, alcohol consumption, and not breastfeeding.[3] Other risk factors include early menarche (< 12 years), late menopause (> 55 years), a family history of breast cancer, inherited mutations in *BRCA1* and *BRCA2* genes, and mammographically dense breasts (compared to least dense).

1.1.2 Treatment regimens for different subtypes of breast cancer

Luminal tumors occupy the greatest population (60-80%) of breast cancer cases in developed countries[3]. For HR-positive breast cancer, the main treatment is endocrine therapy,

which works by blocking the effects of hormone or decreasing the hormone level. Currently available drugs include tamoxifen (a prodrug blocking the binding of estrogen to estrogen receptor); aromatase inhibitors (suppress the conversion of androgen to estrogens); luteinizing hormone-releasing hormone analogs (suppress the production of hormone from the ovary); and fulvestrant (a selective estrogen receptor degrader).[4]

For HER2-postive breast cancer, several molecular targeted agents have been approved to be used alone or in combination with standard chemotherapy. These drugs include trastuzumab (anti-HER2 monoclonal antibody); pertuzumab (anti-HER2 monoclonal antibody with a different binding site on HER2 than trastuzamab); and lapatinib (a dual tyrosine kinase inhibitor that interrupts both HER2 and epidermal growth factor receptor pathways). With the implementation of HER2-targeted therapies, the median overall survival (OS) of patients with HER2-positive advanced breast cancer has increased substantially from approximately 20 months to about 5 years.[5]

Triple negative breast cancer is more aggressive and difficult to treat than HR-positive and HER-positive breast cancer. For triple negative breast cancer, standard chemotherapy remains the major way of treatment. In light of the suboptimal treatment outcome with chemotherapy, new targeted therapies for triple negative breast cancer are badly needed.

1.1.3 The involvement of GPCRs in breast cancer

G-protein-coupled receptors (GPCRs) have been known to play critical roles in the neoplastic transformation of many cancers. Important functions of GPCRs include the regulation of cellular motility, growth and differentiation, and gene transcription, three factors essential to

the biology of cancer.[6] Protease-activated receptor 1 (PAR1), a GPCR, is overexpressed in breast cancer and is responsible for development of metastases in breast cancer patients.[7] PAR1 is also known to promote growth and invasion by promoting detachment and migration of the epithelial cancer cells.[8] Another GPCR, GPR116, plays an important role in cell adhesion and is a novel regulator for breast cancer metastasis.[9] Interestingly, our laboratory also demonstrated the involvement of an important GPCR in breast tumorigenesis.

1.2 AGTR1 and breast cancer

Our laboratory had identified a subpopulation of breast cancer that harbor elevated expression of angiotensin II receptor type I (AGTR1).[10] By utilizing MetaCOPA (MetaAnalysis and Cancer Outlier Profile Analysis) method, we analyzed 31 breast cancer profiling datasets, consisting of 3,157 microarrays. The "outliers" were defined as genes with the most dramatic overexpression in a subset of tumors, while "meta-outliers" were genes identified in statistically significant fraction of datasets. We identified 159 significant meta-outliers ($P<1E-5$), of which about 20 genes were identified as outliers in most of the datasets examined.

Among all human genes represented in the analysis, ERBB2 was the most significant meta-outlier, identified in 21 of 29 independent datasets. Based on the knowledge that ERBB2 and genomic neighbors are coamplified and coexpressed in breast cancer, we observed a clear coexpression pattern of the 17q meta-outliers.

The next most consistently scoring outlier, excluding ERBB2 and genomic neighbors, was AGTR1, the gene encoding angiotensin II receptor type I. AGTR1 was identified as an outlier in

15 out of 22 datasets (68%; P = 2.0E-18). The microarray data clearly indicated that AGTR1 is highly overexpressed in a subset of tumors relative to normal tissue and that high overexpression occurs exclusively in a subset of estrogen receptor-positive (ER^+) tumors. Furthermore, a coexpression analysis of AGTR1 and ERBB2 revealed that the overexpression of these two receptors in breast cancer is mutually exclusive (Figure 2).

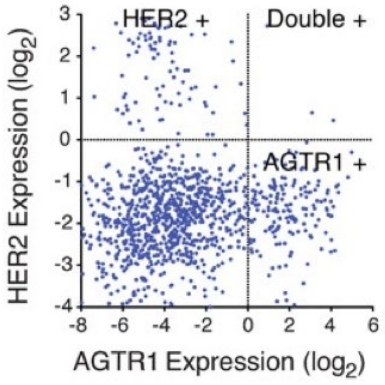


Figure 2 - The mutual exclusivity of AGTR1 and HER2 expression in breast cancer.

1.2.1 Angiotensin II and angiotensin II receptor type I (AGTR1)

Angiotensin II (Ang II) is an octapeptide hormone (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) formed from enzymatic cleavage of angiotensinogen to angiotensin I (Ang I) by the aspartyl protease renin, followed by conversion of Ang I to Ang II by angiotensin converting enzyme (ACE).[11] Ang II has important functions in maintenance of salt-water balance, blood pressure control, aldosterone secretion and effects on the central nervous system, such as thirst sensation and regulation of sympathetic outflow.[12,13]

Most of the physiological effects of Ang II are mediated through angiotensin II receptor type I (AGTR1), which are widely distributed in all organs, including liver, adrenals, brain, lung,

kidney, heart, and vasculature.[14] AGTR1 is a member of the rhodopsin family of G-protein coupled receptors (GPCRs), possessing an extracellular N-terminus, an intracellular C-terminus, and seven highly hydrophobic transmembrane α-helices (H1-7), which are connected by three extra- and intracellular loops.[15] The extracellular domain of the receptor is characterized by three glycosylation sites, while G protein interactions occur on the transmembrane domain at the N-terminus and the first and the third extracellular loops.[14] Upon Ang II stimulation, the Gα subunit binds to the core region of active GPCRs via the opened cytosolic cavity. This interaction triggers nucleotide exchange of Gα from GDP to GTP and dissociation of Gα from Gβγ. The separated subunits trigger downstream signaling cascades, including second messenger generation, activation of small G proteins and cytoplasmic tyrosine kinases, regulation of ion channels or transactivation of growth factor receptors.[12]

Stimulating AGTR1 is able to activate various G proteins, such as $G\alpha_{q/11}$, $G\alpha_{i/o}$, or $G\alpha_{12/13}$.[16] $G\alpha_{q/11}$ protein induces the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) into the second messengers inositol triphosphate (IP_3) and diacylglycerol (DAG) by activating phospholipase Cβ. IP_3 triggers intracellular Ca^{2+} mobilization by binding and opening its calcium channel receptor. Also, in cooperation with DAG, Ca^{2+} can activate various isoforms of protein kinase C (PKC).[12,17] On the other hand, the signal transduction through $G\alpha_{i/o}$ and $G\alpha_{12/13}$ proteins lead to inhibition of adenylyl cyclase, regulation of L- and T-type Ca^{2+} channels and activation of phospholipase D, Rho GTPase and Rho kinase.[18]

1.2.2 AGTR1-positive breast cancer

In our study, the ectopic overexpression of AGTR1 in primary mammary epithelial cells, combined with angiotensin II stimulation, led to a highly invasive phenotype that can be attenuated by the AGTR1 antagonist losartan.[10] Furthermore, losartan reduced tumor growth by 30% in AGTR1-positive breast cancer xenografts. This study provides a rationale for a clinical trial that includes losartan in the treatment of breast cancer patients with AGTR1-positive tumors.

1.3 GPCR and NF-κB signaling

GPCRs that couple to $G\alpha_{q/11}$ protein are known to activate downstream protein kinase C (PKC) isoforms. It has been clear that PKC activation by certain GPCRs is a prerequisite for subsequent NF-κB activation. Before unraveling the connectivity of GPCR to NF-κB signaling, we will take a brief review on the features of NF-κB family of transcription factors, and their mechanism.

1.3.1 The family of NF-κB transcription factors

NF-κB is an inducible and ubiquitously expressed transcription factor for genes involved in immune and inflammatory responses, cell survival, cell adhesion, differentiation, and growth.[19] Active NF-κB complexes are dimers of various combinations of the Rel family of polypeptides consisting of p50 (NF-κB1), p52 (NF-κB2), c-Rel, RelA (p65), and RelB that share N-terminal

homology with the v-Rel oncogene.[20] The Rel homology domain (RHD) is 300 amino acids and has three functions: sequence-specific DNA binding, dimerization, and inhibitory protein binding. Following the RHD is a nuclear localization sequence (NLS). Among the Rel family proteins, p50 was generated from precursor protein p105 (NFKB1), while p52 was processed from precursor protein p100 (NFKB2). Moreover, RelA, RelB, and c-Rel are synthesized as mature proteins with transcription transactivation domains (TADs). The two archetypal dimers are p50:p65 and p52:RelB, although other combinations exist with distinct functions.[21]

1.3.2 Canonical and non-canonical NF-κB signaling

In unstimulated cells, NF-κB dimers are inactive, being sequestered in the cytoplasm by interaction with inhibitory proteins named IκBs (inhibitors of NF-κB).[19] The first step of NF-κB activation involves post-translational modification of IκB inhibitors. This occurs by a canonical pathway and a non-canonical pathway. For the canonical pathway, a kinase complex called IκB kinase (IKK) specifically phosphorylates IκB proteins leading to their degradation.[22] This allows NF-κB dimers to translocate into the nucleus and induce gene expression. The cytosolic IKK holoenzyme contains a regulatory subunit, IKKγ (also NEMO, NF-κB essential modifier), and two kinase subunits, IKKα and IKKβ. IKKγ is a non-catalytic, regulatory subunit that links IKKα and IKKβ into a holo-complex and is required for ubiquitination reactions that trigger oligomerization and signaling (Figure 3).

The rapid and irreversible destruction of IκB proteins in the proteasome is triggered by phosphorylation and ubiquitination. In the canonical pathway, IKKβ phosphorylates IκBα on Ser

32 and Ser 36, or IκBβ on Ser 19 and Ser 23. Phosphorylated IκBs are then polyubiquitinated by $SCF^{\beta\text{-}TrCP}$ E3 ubiquitin ligases, leading to their proteasomal degradation.

By contrast, the non-canonical pathway utilizes a dimer of IKKα to phosphorylate p100 on Ser 176 and 180 that results in proteasomal processing to p52. p100 is mainly complexed with RelB to keep nascent p52 and RelB in the cytoplasm. Instead of IKKβ, IKKγ, or the classical IKK complex, phosphorylation of p100 requires the NF-κB-inducing kinase (NIK). NIK phosphorylates and activates IKKα, which causes the phosphorylation of cytoplasmic p100:RelB by IKKα, finally leading to ubiquitination and proteolysis of the C-terminal domain and nuclear translocation of p52:RelB heterodimer.[23]

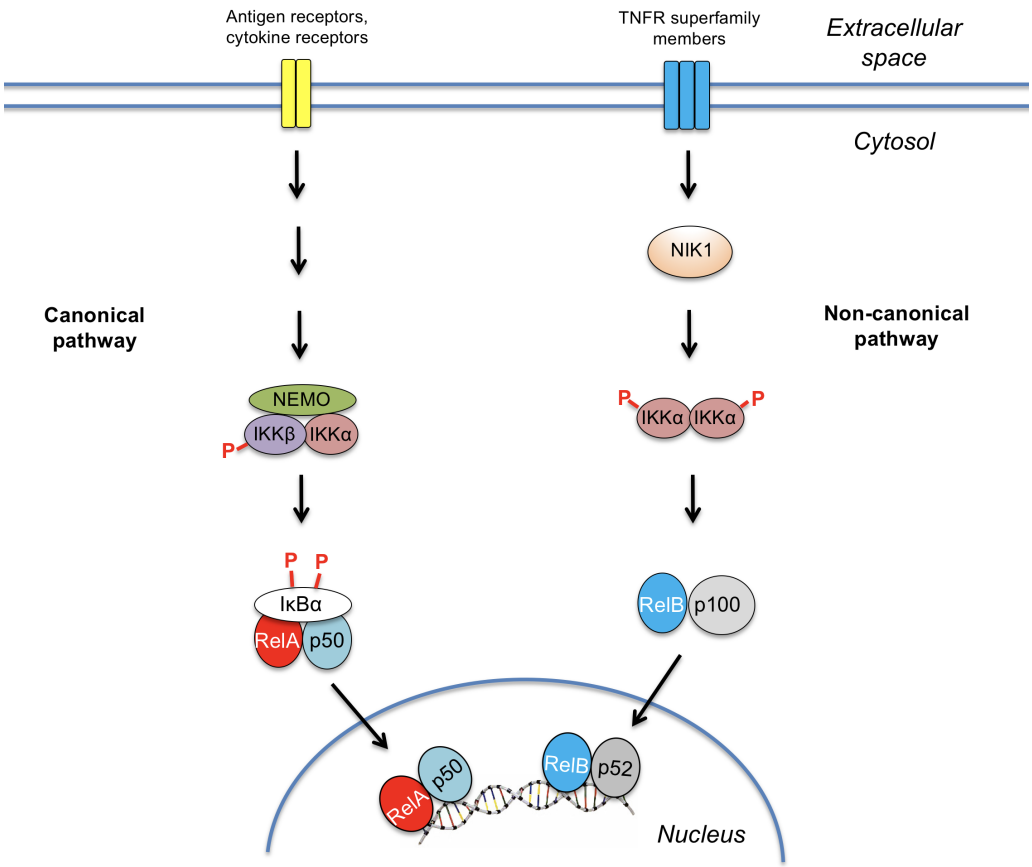


Figure 3 - The Canonical and noncanonical NF-κB pathways.

1.3.3 The role of NF-κB

The physiological role of NF-κB is best described in the immune system. Knockout mice for the RHD proteins show predominantly immunological defects.[24] Canonical pathway inducers, mainly p50:RelA dimers, include all sorts of proinflammatory cytokines such as tumor necrosis factor (TNF) and interleukin-1 (IL-1), conventional humoral and cellular antigens, pathogen-associated molecular patterns (PAMPs) including many bacterial and viral products, cell-bound and soluble immune mediators, and effector molecules. These factors potently activate NF-κB in lymphocytes, macrophages, and dendritic cells and other minor immune cell types.[25] The NF-κB-induced genes include a variety of chemokines, cytokines, adhesion molecules, inflammatory mediators, and apoptosis inhibitors, granting NF-κB an essential role in global immunity.[26]

The non-canonical pathway of NF- κB activation does not respond to canonical pathway inducers. While the canonical pathway responds rapidly to a large variety of immune receptors, the non-canonical pathway only responds to a subset of receptors.[27,28] Many of these inducers are a subgroup of the tumor necrosis factor receptor (TNFR) superfamily, including BAFFR, CD40, RANK, 4-1BB, HVEM, OX40, GITR, Fn14, TNFR2, and CD30, each of which likely relates to a different function of the non-canonical pathway.[24,29]

1.3.4 GPCRs induce NF-κB activity

The GPCR family is the largest family of cell membrane receptors, including over 800 know members. Upon receiving the extracellular signals, GPCRs can activate multiple intracellular signaling pathways that include those for ERK, Akt, JNK, MAPK, STAT, and NF-κB.[30,31] There

are several studies suggesting that GPCRs and their ligands can activate NF-κB in cancer cells, including Ang II/AGTR1-mediated and NF-κB-dependent proliferation of gastric cancer cells,[32] bradykinin/B2 receptor-mediated migration of chondrosarcoma cells,[33] and adenosine/A3 receptor-mediated repression of colon carcinoma cells.[34]

Although the actual mechanisms underlying the Ang II-dependent NK-κB activation was unclear, it has long been considered that the activation of canonical NF-κB pathways requires proximal stimulation of PKC.[35] Having this concept in mind, we and others recognized a parallel theme in the antigen-responsive NF-κB activation in lymphocytes, leading to the discovery of the novel mediators that connect GPCRs and NF-κB signaling.

1.4 The CBM Signalosome

Our laboratory and others previously described a similar signaling pathway that mediates antigen-induced lymphocyte proliferation by linking T or B cell receptor-dependent PKC activation to the stimulation of NF-κB.[36] The pathway includes three major signaling components: CARMA1 [caspase recruitment domain (CARD) 11/Bimp3], Bcl10 [B-cell CLL/lymphoma 10], and MALT1 [mucosa-associated lymphoid tissue lymphoma translocation protein1].[37] Together, these three proteins constitute the CARMA/Bcl10/MALT1 (CBM) signalosome, which plays a critical role in regulating NF-κB activation both in normal physiologic processes and pathophysiologic settings (Figure 4).

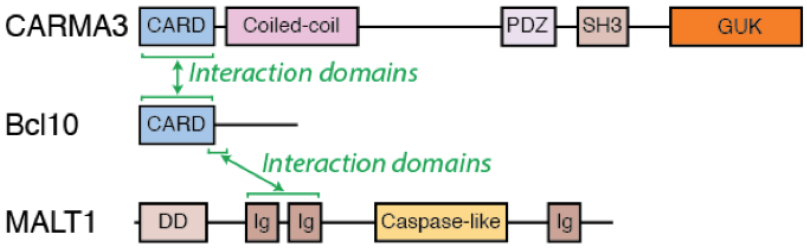


Figure 4 - Schematic representation of the three components of CBM signalosome.

Green arrows represent the domains that can interact with each other. CARD: caspase activation and recruitment domain; GUK: guanylate kinase-like domain; DD: death domain.

1.4.1 CARMA1, what we have learned from lymphocytes

In 2000, a novel CARD-containing and Bcl10-interacting protein, CARD9, was identified, showing ability to stimulate NF-κB activation.[38] A subsequent *in silico* study led to the identification of a family of proteins including CARMA1/CARD11, CARMA2/CARD14, and CARMA3/CARD10.[39-41] CARMA1 is expressed exclusively in lymphocytes and a few related immune cells, and is an essential component of the antigen-induced NF-κB signaling pathway in T cells. In addition to its role in T cell receptor (TCR) signaling, CARMA1 can also mediate natural killer (NK) cell immunoreceptor tyrosine-based activation motif (ITAM)-induced signals, which is important for proper NK cell function.[42]

Upon the activation of the TCR, protein kinase C-θ (PKCθ) is recruited by CARMA1 to a receptor-proximal complex associated with lipid rafts.[43] Then PKCθ is activated by elevated second messenger diacylglycerol and phosphorylates CARMA1 in the linker domain, which is essential for NF-κB activation.[44,45] PKCθ can also be activated by 3-phosphoinositide-dependent kinase (PDK)-1-dependent phosphorylation. In this case, PDK-1 binds to PKCθ and recruits the

IKK complex, at the same time interacting with CARMA1 to recruit Bcl10 and MALT1, subsequently promoting NF-κB activation.[46]

In B cells, the stimulation of B cell receptor (BCR) triggers the recruitment of protein kinase C-β (PKCβ), the CBM signalosome, and the IKK complex into lipid rafts, leading to the phosphorylation of CARMA1 by PKCβ.[47] In both B and T cells, CARMA1 is supposed to exist in an autoinhibited conformation through intramolecular interactions involving multiple regions of the protein. Phosphorylation of CARMA1 in the linker region will overcome the intramolecular interactions and releases the autoinhibition, although details of the conformational change remain to be elucidated (Figure 5).

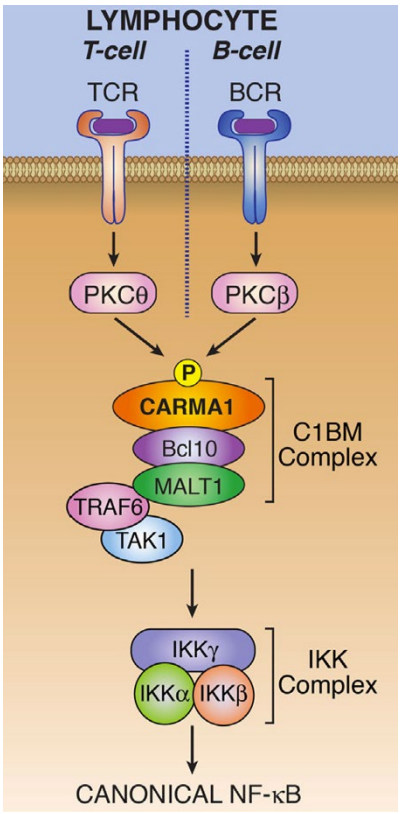


Figure 5 - The CBM signalosome-dependent NF-κB activation in lymphocytes.

1.4.2 CARMA3, a CARMA homologue expressed in non-immune cells

CARMA1 has been shown to be expressed mainly in lymphoid tissues, while CARMA3 is widely expressed outside of immune system.[39,40,48] Subsequent bioinformatic analysis revealed that CARMA3 is actually expressed in almost all tissue types, including kidney, heart, brain, liver, and other tissues of ectodermal, mesodermal, and endodermal origins. The *CARMA1* and *CARMA3* genes encode highly similar proteins: the CARD and coiled-coil domains share approximately 60% and 50% sequence identity respectively with one another, while the PDZ, SH3 and GUK domains share approximately 20-30% identity. Given the similarities between CARMA1 and CARMA3, in 2007, we and two other groups simultaneously investigated and demonstrated that the CARMA3-containing CBM complex functions downstream of specific GPCRs that are able to stimulate PKC, further activating the canonical NF-κB pathway (Figure 6).[49-51] Our own group's study proved the essential role of CARMA3, Bcl10, and MALT1 in the AngII-dependent activation of canonical NF-κB pathway outside of the immune system,[49] while other groups focused on the lysophosphatidic acid receptor (LPAR) family,[50,51] and the receptor for endothelin-1 (EDNRA; also known as ET_AR).[51] Since the initial discovery in 2007, the list of GPCRs that utilize CARMA3-containing CBM complex as a signaling mediator has been greatly increased, including chemokine receptor type 2 (CXCR2),[52] CXCR4,[53] platelet-activating factor receptor (PTAFR; also known as PAF-R),[54] and protease-activated receptor 1 (F2R; also known as PAR1).[55]

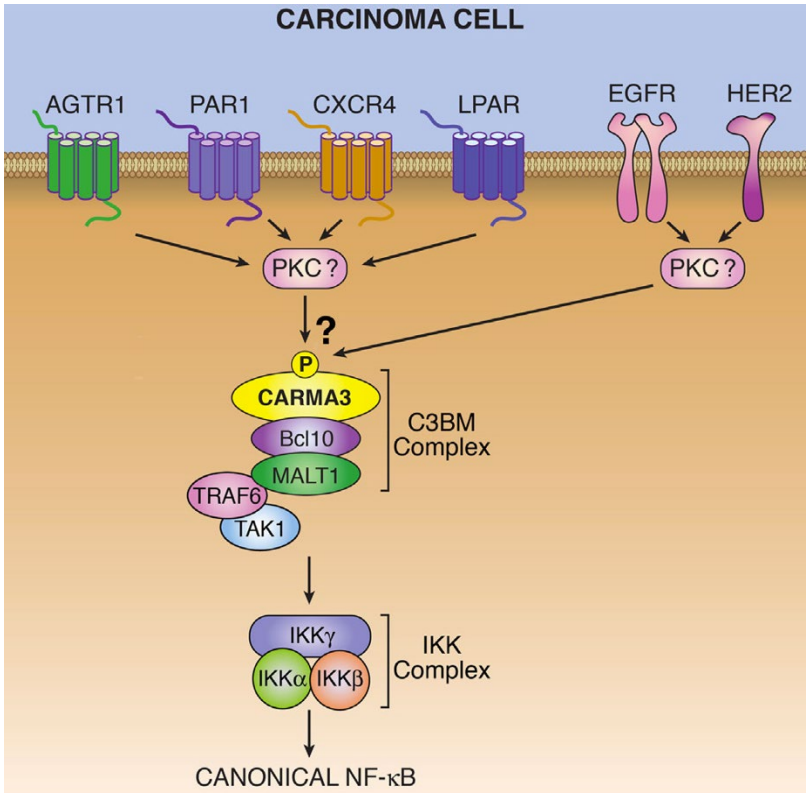


Figure 6 - The CBM signalosome-dependent NF-κB activation in carcinomas.

1.4.3 MALT1, a mediator with dual roles within the CBM signalosome

1.4.3.1 MALT1 is a functional protease

MALT1 is a ubiquitously expressed protease which resides in the cytoplasm as a catalytically inactive Bcl10-bound proenzyme. It is recruited into the CBM complex upon the stimulation of certain GPCRs or receptor tyrosine kinases.[37] The MALT1 gene was originally identified as the target of recurrent translocation in MALT lymphomas.[56-58] The following *in silico* studies revealed that MALT1 shares homology with caspases and metacaspases, thus falling into a category of caspase-like proteases termed paracaspases.[59,60] In addition to structural homology, several other aspects of MALT1 function are shared with caspases, such as cysteine-dependent

protease activity,[61,62] a requirement for dimerization,[63,64] and the capacity to auto-process.[65,66] Monomeric MALT1 is inactive and requires dimerization via the caspase-like protease domain for enzymatic activity.[63,64,67] It is interesting that MALT1 has recently been demonstrated to undergo autoprocessing. Unlike caspases, MALT1 cleaves itself between the N-terminal death domain and the first Ig domain, which seems essential for MALT1-dependent NF-κB activation.[65,66]

Several important differences still exist in spite of the similarities between MALT1 and caspases. One major difference falls in their substrate specificity. While caspases are aspartic acid-specific proteases, MALT1 cleaves its substrates after arginine residues.[61,62,67] The substrates for MALT1 protease include RelB[68] and A20,[61] negative regulators of NF-κB, as well as MALT1 autoprocessing.[65,66] RelB plays a critical role in inhibiting the canonical NF-κB pathway by forming transcriptionally inactive complexes with RelA and c-Rel.[68] MALT1-dependent RelB cleavage causes RelB proteasomal degradation, thus promoting RelA-, and c-Rel-depenedent NF-κB activation.[68] On the other hand, MALT1-dependent cleavage of the deubiquitinating enzyme (DUB) A20 relieves its inhibitory role in NF-κB activation.[61] It was proposed that A20 negatively regulates the scaffolding function of MALT1 by removing the MALT1 polyubiquitination.[69] However, the regulatory role of A20 on MALT1 function still requires further investigation.[70]

1.4.3.2 The scaffolding role of MALT1

Another important difference between MALT1 and caspases is the regulation of MALT1 function, which depends on posttranslational modification by ubiquitination. Upon the stimulation of upstream receptors, MALT1 is ubiquitinated within the Ig3 domain and its C-terminal part by either K63-linked polyubiquitination or monoubiquitination. MALT1 polyubiquitination mediated by TRAF6 is essential for the activation and physical recruitment of the IKK complex, which

phosphorylates IκB and leads to its proteasomal degradation and the nuclear translocation of NF-κB.[71-73] This K63-linked polyubiquitin chains of MALT1 are thought to provide docking sites for the adaptor protein TAB2, which forms a complex with the IKK-activating kinase TAK1,[74] leading to phosphorylation and activation of IKKβ. Moreover, the K63-linked ubiquitin chains may serve to physically recruit the IKK complex via its regulatory subunit IKKγ.[71,75] MALT1 also associates with the linear ubiquitination chain assembly complex LUBAC, but the consequences of this interaction for IKK activation remain incompletely understood.[76]

1.4.3.3 MALT1 deregulation and disease

Deregulations of MALT1 function are associated with the development of various diseases, including immunodeficiency,[77-79] enteropathy, lymphoid malignancies or lymphoproliferative disease.[80,81] Deregulated MALT1 function also plays a wider a role in allergies,[82,83] psoriasis,[84,85] and a variety of non-lymphoid cancers, such as glioblastoma,[86] lung cancer[87], breast cancer,[88] melanoma,[89] and other carcinomas.[90,91] Some studies suggested that MALT1 expression *per se* might play a role in promoting carcinogenesis.[86,89] Since MALT1 is a druggable protease, the development of MALT1 protease inhibitors may benefit the treatment of patients with such malignancies.

1.4.3.4 The development of MALT1 protease inhibitor

The first MALT1 inhibitor identified was z-VRPR-fmk, a modified tetrapeptide based on the optimal substrate Val-Arg-Pro-Arg of the *Arabidopsis thaliana* metacaspase AtmC9, conjugated to fluoromethyl ketone (fmk).[62] This modified peptide irreversibly blocked MALT1

protease activity and efficiently inhibited T cell activation and IL-2 secretion in Jurkat T cells and in human antigen specific cytotoxic T lymphocytes (CTLs).[62]

Several recent studies have reported the development of small molecule MALT1 inhibitors acting as active site or allosteric inhibitors.[92-96] Nagel and colleages have identified three phenothiazine derivatives (mepazine, thioridazine, and promazine) as highly specific, non-competitive and reversible MALT1 inihibitors.[92] A concurrent study by Fontan and colleagues has identified the compound MI-2 as a selective MALT1 inhibitor, which, in contrast to phenothiazine derivatives, binds irreversibly to the active site of MALT1.[93] These small molecule MALT1 inhibitors have been shown to be effective against activated B-cell-like (ABC) diffuse large B-cell lymphoma (DLBCL) in xenograft models. The successes of MALT1 inhibitors against lymphoma has encouraged the study in the potential of MALT1 protease inhibitors as a novel therapeutic for solid tumors.

1.5 Hypothesis

Knowing that AGTR1 and HER2 are mutually exclusive among breast cancer population, and AGTR1 is a negative prognostic factor in breast cancer, we are interested in the influence of AGTR1 overexpression on breast tumorigenesis. Furthermore, our previous studies of the CBM signalosome in nonimmune cells and endothelial cells suggested its involvement in AGTR1-dependent NF-κB activation. Therefore, we hypothesize that *AGTR1-dependent NF-κB activation is mediated by the CBM signalosome and contributes to the pathogenesis of a subset of breast cancers*.

2.0 Results - Part 1

This part of study was published on *Cancer Research* in March, 2018. The title of this paper is "The CRMA3-Bcl10-MALT1 signalosome drives NF-κB activation and promotes aggressiveness in angiotensin II receptor-positive breast cancer".[97]

2.1 AGTR1 overexpression defines a subset of luminal breast cancers with poor prognosis

Based on MetaCOPA analysis of 31 independent microarray datasets, we previously demonstrated that AGTR1 overexpression defines a subset of breast cancers.[10] By using kernel density plot analysis, we evaluated the distribution pattern of AGTR1 mRNA in The Cancer Genome Atlas (TCGA) database, and the result showed that about 19.5% of cases fell within a group characterized by distinct high levels of AGTR1 mRNA (Figure 7A). Importantly, we found that AGTR1 and HER2 overexpression are mutually exclusive in the cases from the TCGA collection (Figure 7B). When sorted by PAM50 subtype, $AGTR1^+$ cases clustered within the ER^+ (luminal A and luminal B) subgroups (Figure 7C).

To identify whether AGTR1 overexpression functionally drives protumorigenic behavior, we analyzed the TCGA collection of invasive ductal carcinomas and found that AGTR1 overexpression is highly associated with axillary node metastases (Figure 7D). Moreover, gene

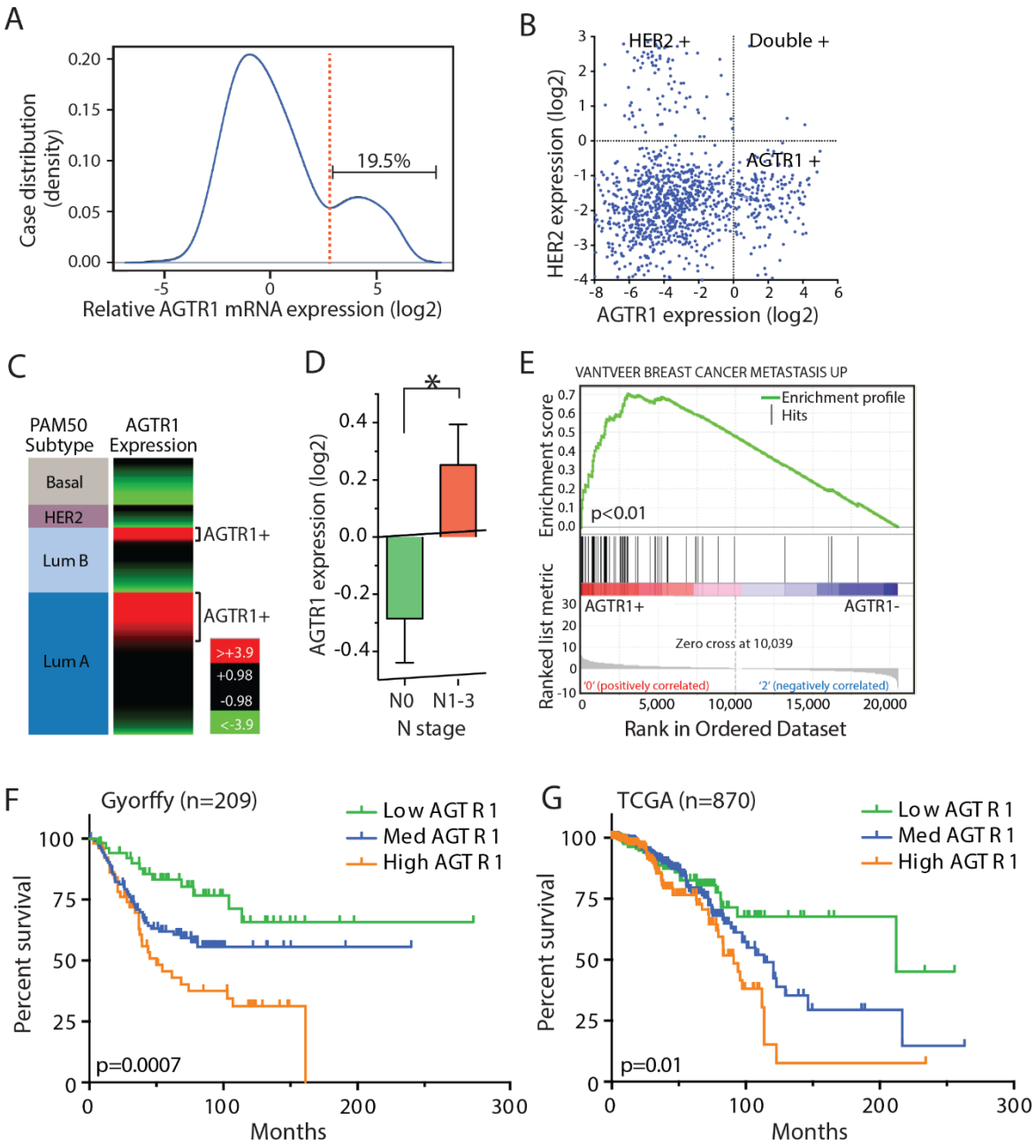


Figure 7 – AGTR1 is expressed in a subset of breast cancers and is associated with aggressive disease.

A, Kernel density plot analysis for AGTR1 mRNA expression in TCGA invasive breast cancer. B, Scatter plot of HER2 and AGTR1 mRNA expression in the same TCGA cases. C, Heatmap subcategorization of AGTR1+ cases based on PAM50 subtype analysis. D, Based on TCGA data, AGTR1 overexpression as a function of N stage (nodal status) for invasive ductal carcinoma. E, GSEA performed on AGTR1-stratified breast cancer in the TCGA collection. F and G, Kaplan-Meier survival curves based on AGTR1 expression.

set enrichment analysis (GSEA) of TCGA cases showed that AGTR1^{+} tumors displayed dramatic enrichment for genes linked to poor prognosis and development of distant metastasis within 5 years (Figure 7E). Ultimately, Kaplan-Meier plots generated from both the TCGA datasets and from the Gyorffy meta-analysis showed that AGTR1 overexpression strongly correlates with reduced breast cancer survival (Figure 7F and G).

2.2 AGTR1 overexpression directs NF-κB-dependent gene expression reprogramming in breast cancer cells

In light of the mutual exclusivity of AGTR1 and HER2 overexpression in breast cancer, we hypothesized that AGTR1 and HER2 may share a common and redundant downstream signaling pathway. Although each receptor can activate a variety of intracellular signaling pathways regulating cancer progression, both receptors are capable of inducing canonical NF-κB pathway. Therefore, we looked for evidence of AGTR1 overexpression-driven NF-κB activation by specifically investigating NF-κB gene targets that are known to be expressed in epithelial cells and that have been highlighted in breast cancer literature. The result showed that many of these genes were significantly upregulated in AGTR1+ invasive breast cancer from the TCGA collection (Figure 8A), including genes that affect pathogenic processes such as survival and proliferation (BCL2, CCND1, DUSP6, TNFRSF10C), migration and invasion (IL1β, PCSK6, CSF1, JAG1, ZEB1, SAA1), stemness (CD44, BMPR1B, DCLK1, JAG1), and angiogenesis (IL1β, NPY1R, IL6ST/GP130).

In order to select appropriate cell lines for our study, we analyzed endogenous AGTR1 level through the newer expression profiling data of the Cancer Cell Line Encyclopedia (CCLE). The results showed that BT549 is one of the breast cancer cell lines that express highest AGTR1, while ZR75-1 has undetectable AGTR1 mRNA. To imitate acquired AGTR1 overexpression in ZR75-1, we stably transfected this parental cell line with an AGTR1 expression vector, creating ZR75-AGTR1 cell. We also created the negative control cell ZR75-Neo with an empty vector.

In ZR75-1 cell, we found that AGTR1 overexpression alone significantly induced NF-κB activity, which was indicated as increased nuclear translocation of RelA in ZR75-AGTR1, in comparison to the negative control ZR75-Neo cell (Figure 8B and C). This increase in basal nuclear RelA was reflected by expressing a transfected NF-κB-luciferase reporter in ZR75-AGTR1 cell, which showed a >20-fold increase in NF-κB-dependent transcriptional activity (Figure 8D). Although AGTR1 overexpression results in increased endogenous NF-κB activity, stimulating ZR75-AGTR1 with Ang II can cause even greater NF-κB activation, with >100-fold more than the level observed in ZR75-Neo cell (Figure 8D).

To demonstrate the requirement of Ang II on NF-κB activation in $AGTR1^+$ breast cancer cells, we treated ZR75-Neo, ZR75-AGTR1, and BT549 cells with Ang II for different time course. We utilized pIκB as an indicator for NF-κB activation. The result of Western blot analysis showed that there's no detectable pIκB in ZR75-Neo cell after Ang II stimulation, however, ZR75-AGTR1 and BT549 cells displayed an acute NF-κB activation after Ang II treatment (Figure 8E). Interestingly, the level of Ang II-stimulated pIκB in BT549 cell is very similar to that observed in SKBR3, a $HER2^+$ breast cancer cell (Figure 8F).

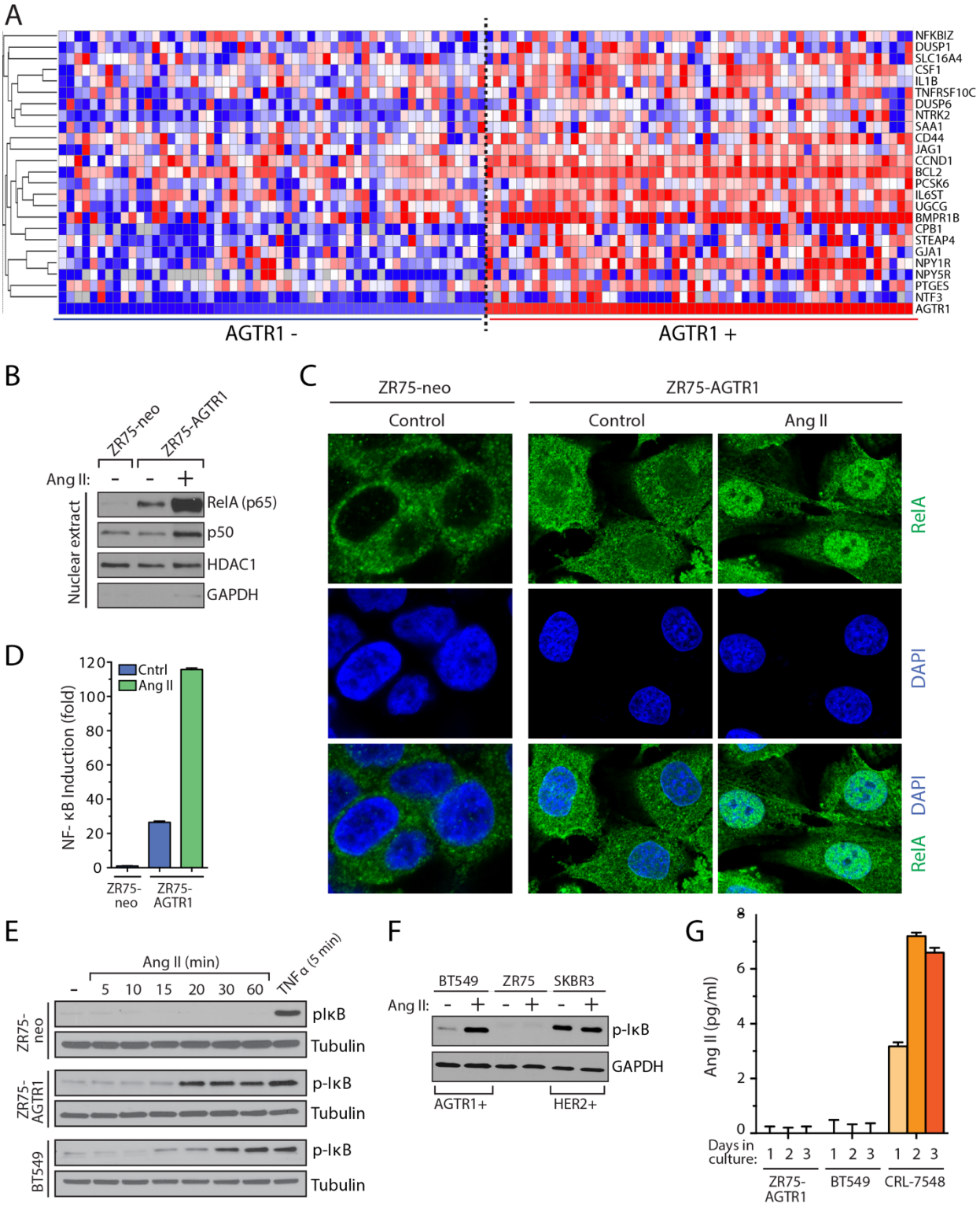

A
NFKBIZ
DUSP1
SLC16A4
CSF1
IL1B
TNFRSF10C
DUSP6
NTRK2
SAA1
CD44
JAG1
CCND1
BCL2
PCSK6
IL6ST
UGCG
BMPR1B
CPB1
STEAP4
GJA1
NPY1R
NPY5R
PTGES
NTF3
AGTR1
AGTR1 -
AGTR1 +
B
ZR75-neo
ZR75-AGTR1
Ang II: - - +
RelA (p65)
p50
HDAC1
GAPDH
Nuclear extract
C
ZR75-neo
ZR75-AGTR1
Control
Control
Ang II
RelA
DAPI
DAPI
RelA
D
NF- κB Induction (fold)
120
100
80
60
40
20
0
Cntrl
Ang II
ZR75-neo
ZR75-AGTR1
E
Ang II (min)
- 5 10 15 20 30 60
TNF α (5 min)
ZR75-neo
pIκB
Tubulin
ZR75-AGTR1
p-IκB
Tubulin
BT549
p-IκB
Tubulin
F
BT549
ZR75
SKBR3
Ang II: - + - + - +
p-IκB
GAPDH
AGTR1+
HER2+
G
Ang II (pg/ml)
8
6
4
2
0
Days in culture: 1 2 3 1 2 3 1 2 3
ZR75-AGTR1
BT549
CRL-7548

Figure 8 - AGTR1 drives NF-κB activity in breast cancer (previous page).

A, Heatmap of expression for key NF-κB- regulated genes in luminal A/B breast cancer cases. B and C, Nuclear translocation of NF-κB subunits (RelA and p50) in ZR75-AGTR1 cells. D, NF-κB luciferase reporter activity with and without Ang II. E, AngII-dependent NF-κB activation in AGTR1^{+} cells. F, NF-κB activity in BT549 cells compared with the HER2+, SKBR3 cell line. G, Ang II levels from conditioned media measured by ELISA.

Additionally, the basal NF-κB activity in AGTR1-overexpressing cells might be induced by an autocrine loop, with cells producing excessive Ang II. To exclude this possibility, we harvested the conditioned media from ZR75-AGTR1 and BT549, and analyzed it with Ang II ELISA (Figure 8G). The result showed that no Ang II was detectable in the conditioned media of ZR75-AGTR1 and BT549 after incubation for up to 3 days.

2.3 The CBM signalosome mediates NF-κB activation in AGTR1^{+} breast cancer cells

Previously our laboratory reported that the CBM signalosome mediates AGTR1-dependent NF-κB activation in endothelial cells.[49,98] Therefore, we tried to investigate whether the same CBM signalosome is involved in NF-κB activation in AGTR1-overexpressing breast cancers. To answer this question, we utilized siRNAs specifically targeting each of the CBM components in ZR75-AGTR1 and BT549 cells. The results showed that transient knockdown of CARMA3, Bcl10, or MALT1 in both cells fully abrogated the p-IκB response (Figure 9A). Importantly, the effect of knocking down the CBM components is equivalent to that of treating cells with losartan or IKKβ

inhibitor, IKK-VI (Figure 9B). These results suggest that the AGTR1-CBM-NF-κB signaling axis also plays an essential role in solid tumor pathogenesis.

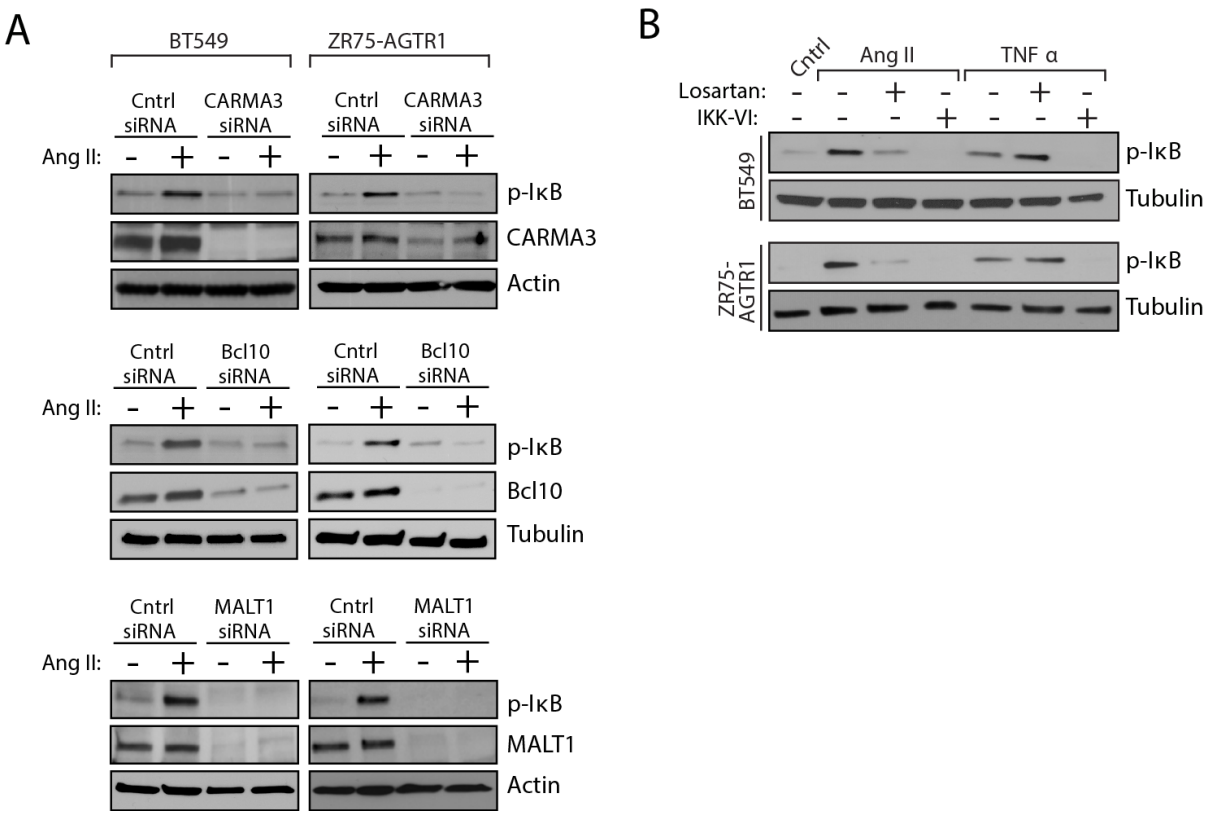


Figure 9 - The CBM signalosome mediates NF-κB activation in AGTR1^{+} breast cancer.

A, Effect of siRNA-mediated knockdown of each individual component of CBM signalosome on Ang II-dependent NF-κB activation. B, Effect of losartan (5μmol/L) or IKK-VI (5μmol/L) on Ang II -dependent NF-κB activation.

2.4 The CBM signalosome critically influences gene expression reprogramming in AGTR1+ breast cancer cells

Our next step was to test the influence of the CBM signalosome NF-κB-dependent gene expression reprogramming by disrupting Bcl10. We knocked down Bcl10 with siRNA in BT549 cell and analyzed the alterations in gene expression using a custom NanoString codeset, which was designed to measure 72 NF-κB regulated mRNAs. We found that 41 out of the 72 genes can be quantified in BT549 cells and clustered within several different groups (Figure 10A). In Group A, many genes are induced by Ang II in BT549 cells, but are not induced in Bcl10-silenced BT549 cells. In Group B, these genes require Bcl10 for both basal and Ang II-induced expression. These results reconfirmed our observation that AGTR1 overexpression mediates both basal and Ang II-responsive NF-κB activity in breast cancer cells.

By utilizing the NanoString PanCancer Progression codeset, we then tested the impact of Bcl10 knockdown on different biological phenomena, including 770 genes important to solid tumor pathogenesis. Ingenuity Pathway Analysis (IPA) showed that Bcl10 knockdown significantly impacted at least three different cancer hallmarks: (1) proliferation and survival, (2) invasion and metastasis, and (3) tumor angiogenesis. To be specific, Bcl10 knockdown causes upregulation of tumor suppressor pathways (PTEN and p53), as well as elevated apoptosis signaling (Figure 10B). On the contrary, Bcl10 knockdown results in decreased activity of growth factor and motility signaling networks (Figure 10C). Ultimately, Bcl10 knockdown inhibited critical angiogenesis pathways, including those for IL6, IL8, and VEGF (Figure 10D).

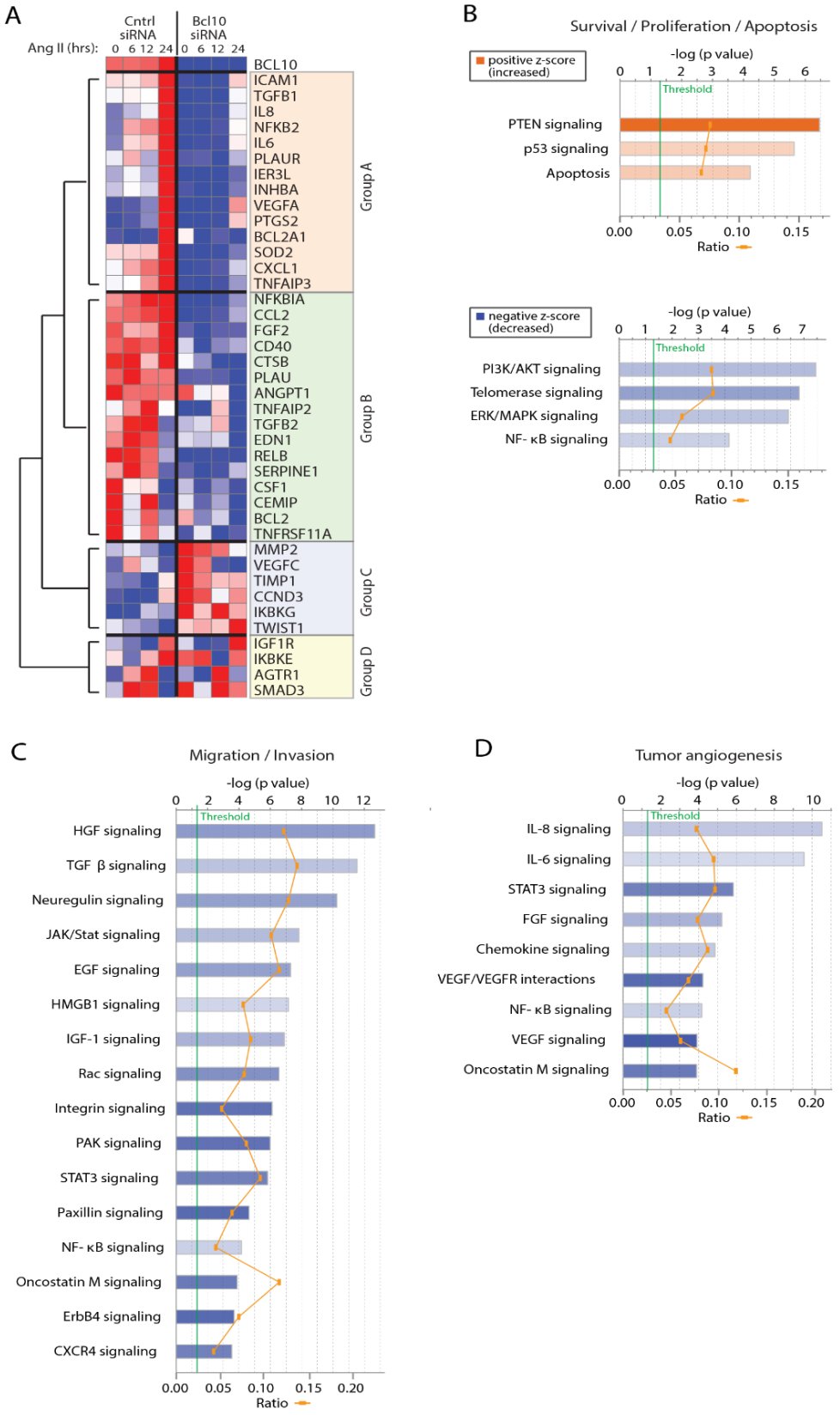

A
Cntrl siRNA
Bcl10 siRNA
Ang II (hrs): 0 6 12 24 0 6 12 24
BCL10
ICAM1
TGFB1
IL8
NFKB2
IL6
PLAUR
IER3L
INHBA
VEGFA
PTGS2
BCL2A1
SOD2
CXCL1
TNFAIP3
NFKBIA
CCL2
FGF2
CD40
CTSB
PLAU
ANGPT1
TNFAIP2
TGFB2
EDN1
RELB
SERPINE1
CSF1
CEMIP
BCL2
TNFRSF11A
MMP2
VEGFC
TIMP1
CCND3
IKBKG
TWIST1
IGF1R
IKBKE
AGTR1
SMAD3
Group A
Group B
Group C
Group D
B
Survival / Proliferation / Apoptosis
positive z-score (increased)
-log (p value)
Threshold
PTEN signaling
p53 signaling
Apoptosis
Ratio
negative z-score (decreased)
PI3K/AKT signaling
Telomerase signaling
ERK/MAPK signaling
NF- κB signaling
C
Migration / Invasion
HGF signaling
TGF β signaling
Neuregulin signaling
JAK/Stat signaling
EGF signaling
HMGB1 signaling
IGF-1 signaling
Rac signaling
Integrin signaling
PAK signaling
STAT3 signaling
Paxillin signaling
NF- κB signaling
Oncostatin M signaling
ErbB4 signaling
CXCR4 signaling
D
Tumor angiogenesis
IL-8 signaling
IL-6 signaling
STAT3 signaling
FGF signaling
Chemokine signaling
VEGF/VEGFR interactions
NF- κB signaling
VEGF signaling
Oncostatin M signaling

Figure 10 - The CBM signalosome mediates gene expression reprogramming in AGTR1$^+$ breast cancer.

A, siRNA-mediated Bcl10 knockdown in BT549 cells and effect on NF-κB gene targets. B, C and D, Ingenuity pathway analysis (IPA) indicates level of significance of change in the indicated pathway.

2.5 The CBM signalosome controls protumorigenic phenotypes in AGTR1+ breast cancer cells

Since Bcl10 knockdown shows an extensive influence on the protumorigenic gene expression signature in AGTR1$^+$ breast cancer cells, we intend to identify which protumorigenic phenotypes are most dependent on the CBM/NF-κB pathway. We first tested the impact of NF-κB inhibition on proliferation, migration, and invasion without Ang II stimulation. Treating ZR75-AGTR1 and BT549 with IKK VI, an IKKβ inhibitor, dramatically abrogated the proliferation of AGTR1+ breast cancer cells (Figure 11A and B). Next, we tested the impact of disrupted CBM signalosome, and found that the proliferation of AGTR1$^+$ breast cancer cells were similarly impaired after CARMA3 or MALT1 knockdown (Figure 11C and D).

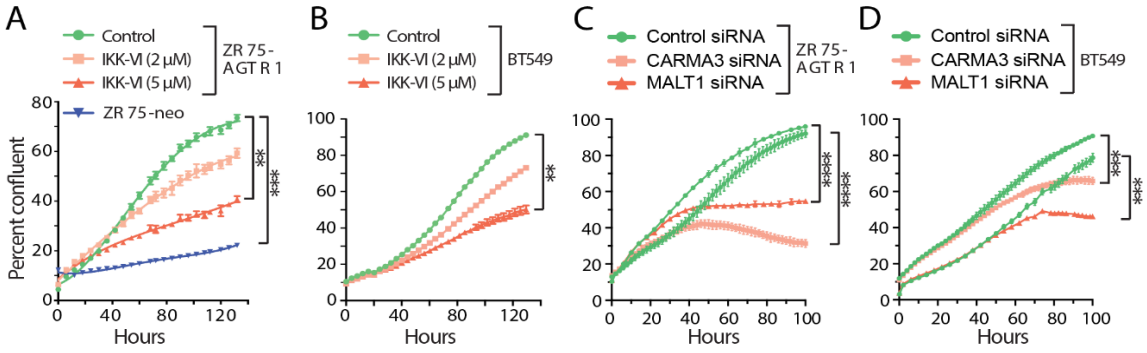


Figure 11 - The CBM/NF-κB pathway is critical for AGTR1-dependent cell proliferation.

(**, $P < 0.01$, ***, $P < 0.001$, ****, $P < 0.0001$)

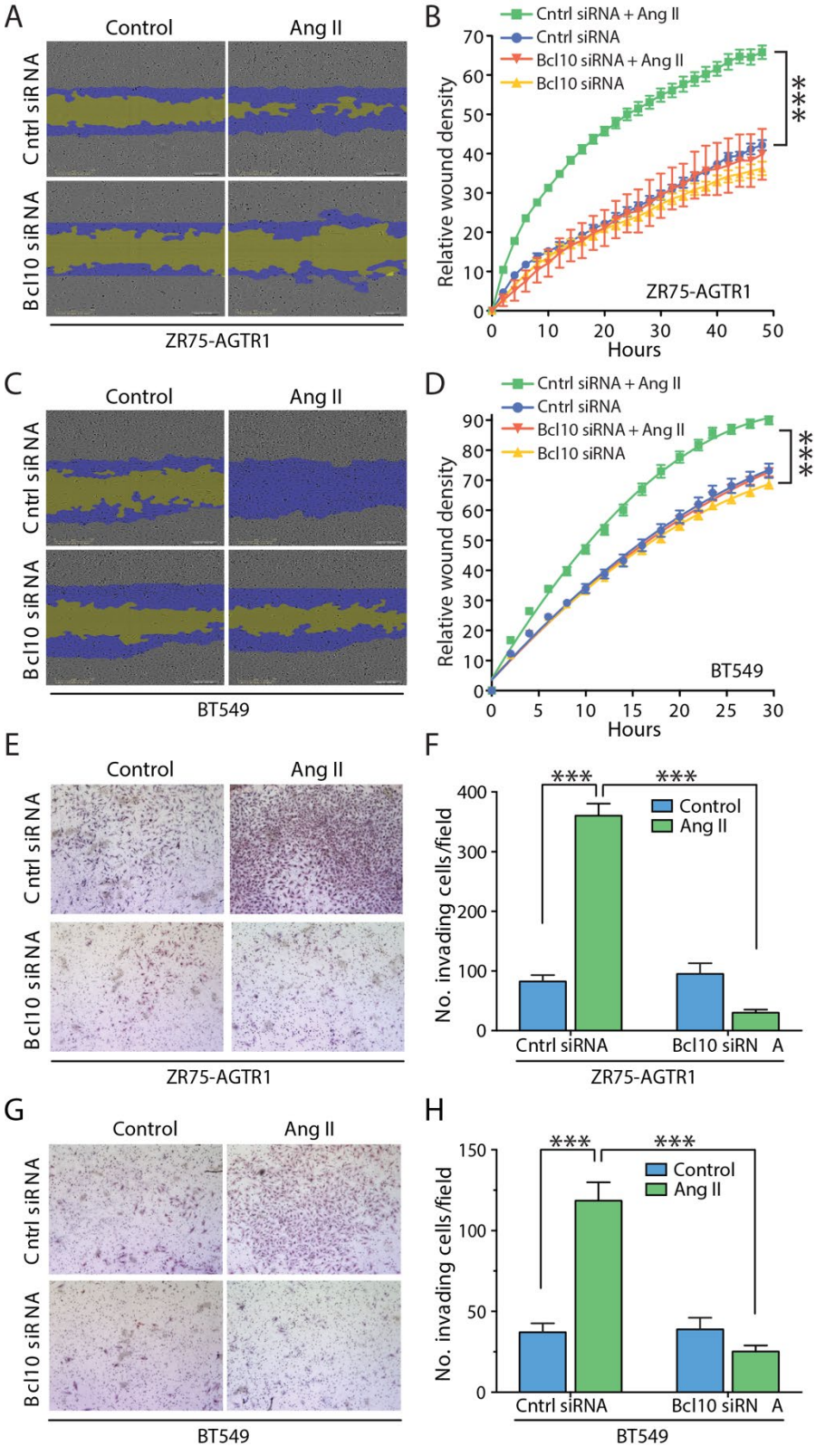

A
Control
Ang II
Cntrl siRNA
Bcl10 siRNA
ZR75-AGTR1
B
Cntrl siRNA + Ang II
Cntrl siRNA
Bcl10 siRNA + Ang II
Bcl10 siRNA
Relative wound density
Hours
ZR75-AGTR1
***
C
Control
Ang II
Cntrl siRNA
Bcl10 siRNA
BT549
D
Cntrl siRNA + Ang II
Cntrl siRNA
Bcl10 siRNA + Ang II
Bcl10 siRNA
Relative wound density
Hours
BT549
***
E
Control
Ang II
Cntrl siRNA
Bcl10 siRNA
ZR75-AGTR1
F
***
***
Control
Ang II
No. invading cells/field
Cntrl siRNA
Bcl10 siRNA
ZR75-AGTR1
G
Control
Ang II
Cntrl siRNA
Bcl10 siRNA
BT549
H
***
***
Control
Ang II
No. invading cells/field
Cntrl siRNA
Bcl10 siRNA
BT549

Figure 12 - The CBM pathway is critical for Ang II-induced cell migration and invasion.

A and B, ZR75-AGTR1 cell migration was monitored in a real-time scratch assay. C and D, BT549 cell migration was monitored in a real-time scratch assay. E and F, ZR75-AGTR1 cell invasiveness was measured with Matrigel-coated Boyden chambers. G and H, BT549 cell invasiveness was measured in Boyden chambers (***, $P < 0.001$).

With Scratch wound assay, we demonstrated that the Ang II-dependent migratory ability of ZR75-AGTR1 and BT549 cells was greatly impaired by knocking down Bcl10 (Figure 12A and B). The Ang II-dependent invasive ability of $AGTR1^+$ breast cancer cells was also severely prohibited after Bcl10 knockdown (Figure 12C and D).

2.6 Bcl10 is required for $AGTR1^+$ tumor angiogenesis *in vivo*

Our gene expression data strongly suggests that the CBM pathway is regulating angiogenic gene signature, so we want to test whether Ang II would induce angiogenesis in and *in vivo* model. To fulfill this purpose, we mixed Ang II-treated breast cancer cells with concentrated, growth factor-depleted Cultrex to create a semisolid suspension, and then implanted them subcutaneously in athymic nude mice. These implanted "plugs" would serve as a matrix for the potential ingrowth of host vasculature over the next 12 – 14 days. Compared with plugs of control ZR75-Neo cells, the plugs in ZR75-AGTR1 cells showed grossly obvious, enhanced vascularization (Figure 13A). We confirmed this observation by quantifying plug hemoglobin content (Figure 13B).

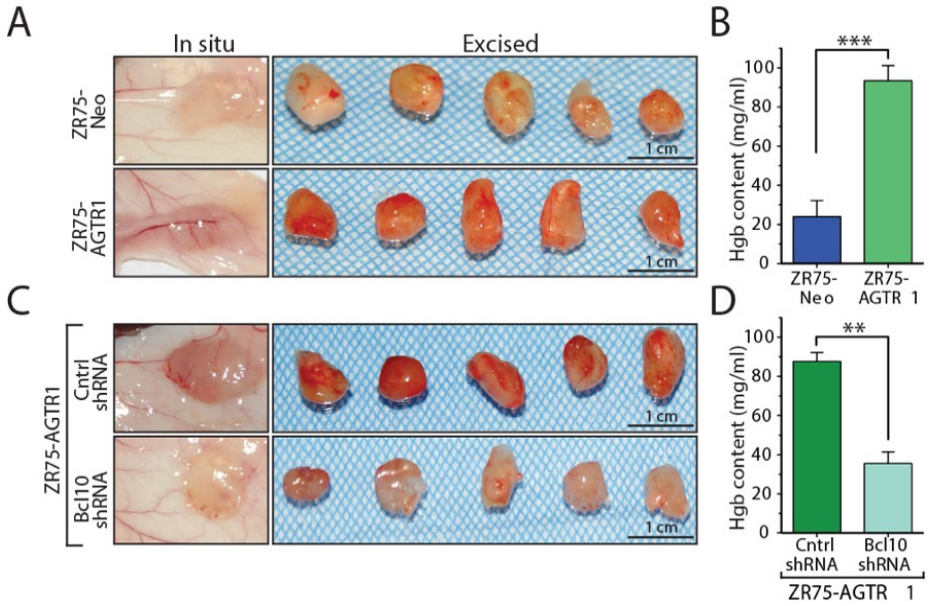


Figure 13 - The CBM pathway is critical for tumor angiogenesis *in vivo*.

A and B, ZR75-AGTR cells were compared to control ZR75-Neo cells for tumor-induced angiogenesis. C and D, Plugs containing ZR75-AGTR1 cells with control versus Bcl10 knockdown (**, $P < 0.01$, ***, $P < 0.001$).

Plugs formed with ZR75-AGTR1 cells engineered to stably express a nontargeting shRNA are similar to those composed of parental ZR75-AGTR1 cells. On the contrary, plugs formed from ZR75-AGTR1 cells expressing Bcl10 shRNA are clearly pale and lacking in vascularization, which was confirmed by measuring hemoglobin content (Figure 13C and D).

2.7 Bcl10 is required for overall AGTR1$^+$ breast cancer growth and host tissue disruption *in vivo*.

Next, tumor xenografts were performed to determine the influence of AGTR1 on tumor growth *in vivo*. We found that xenografts consisting of ZR75-AGTR1 cells grow significantly

faster than those consisting of control ZR75-Neo cells, ultimately producing larger, heavier tumors (Figure 14A-C). Knockdown of Bcl10 via stable shRNA expression dramatically reverses tumor growth, while control shRNA expression has no effect (Figure 14 A-C).

To evaluate tissue invasion, we utilized the chicken chorioallantoic membrane (CAM) model. We layered Ang II-treated ZR75-AGTR1 cells, with or without Bcl10 knockdown, on the surface of the living chicken membranes and observed the resulting interaction between cancer cells and the host tissue after a short, 4-day period. Results showed that ZR75-AGTR1 cells expressing control shRNA induce a vigorous host tissue response, leading to irregular tumor cell/host tissue interface that resembles the invasive front of an aggressive carcinoma (Figure 14 D). In contrast, ZR75-AGTR1 cells expressing Bcl10 shRNA remain completely quiescent on the surface of the CAM and show almost no discernible effects on the host membrane (Figure 14D).

2.8 Conclusion

In this study, we identified a subpopulation of breast cancer aberrantly overexpressing angiotensin II receptor type 1 (AGTR1). Mediated by the CBM signalosome, these AGTR1-positive breast cancers display aggressive phenotypes, such as angiogenesis, proliferation, migration, and invasion (Figure 15). The discovery of the AGTR1/CBM/NF-κB signaling axis not only provides us with novel targets for treating breast cancer but also suggests the potential involvement of other GPCRs in breast tumorigenesis.

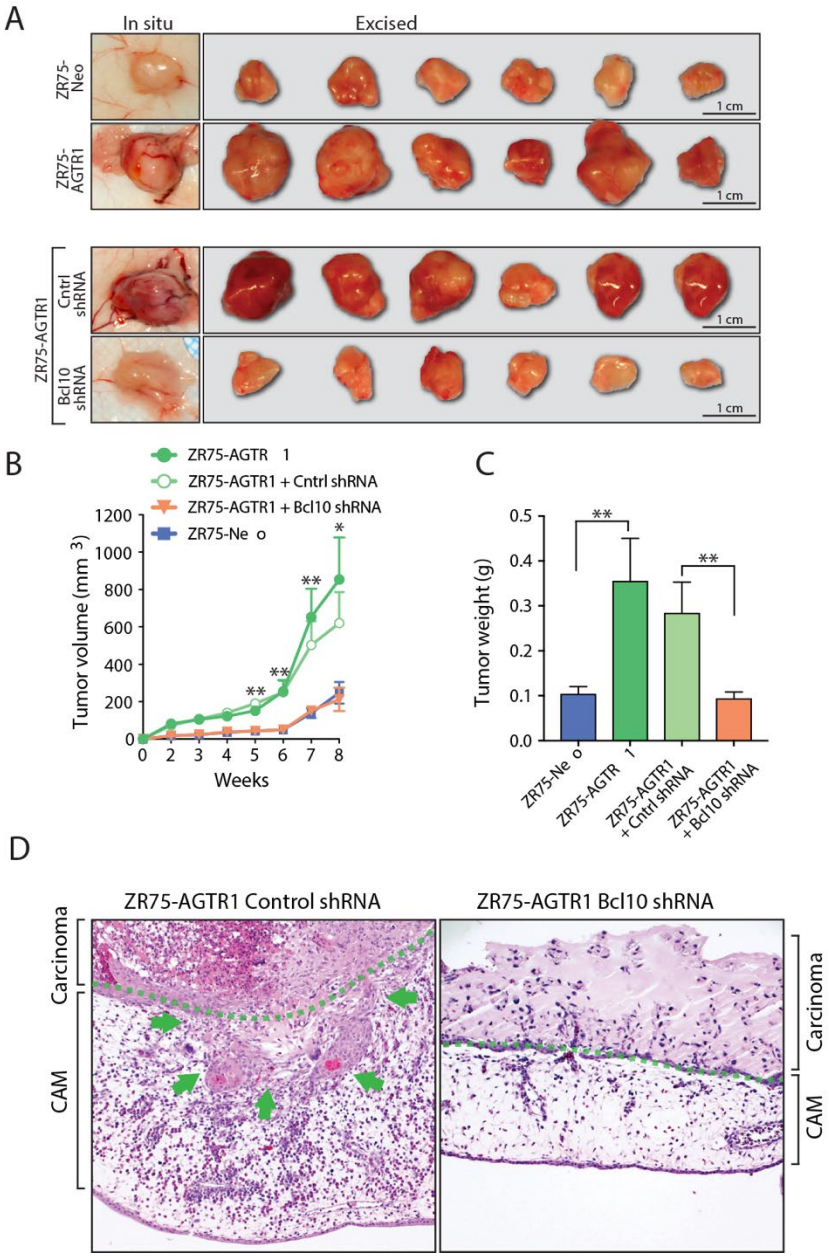


Figure 14 - The CBM pathway is critical for tumor growth and vascularization of AGTR1$^+$ breast cancer.

A-C, Enforced AGTR1 expression in ZR75 cells confers enhanced xenograft growth in vivo, which is fully abrogated by shRNA-mediated Bcl10 knockdown (**, $P < 0.01$). D, Representative photomicrographs (200X) of CAMs after four days of exposure to cancer cells (with or without Bcl10 knockdown) placed on the CAM surface. Green dotted line indicates the level of CAM surface epithelium. Green arrows highlight an area of disruption in the surface epithelium.

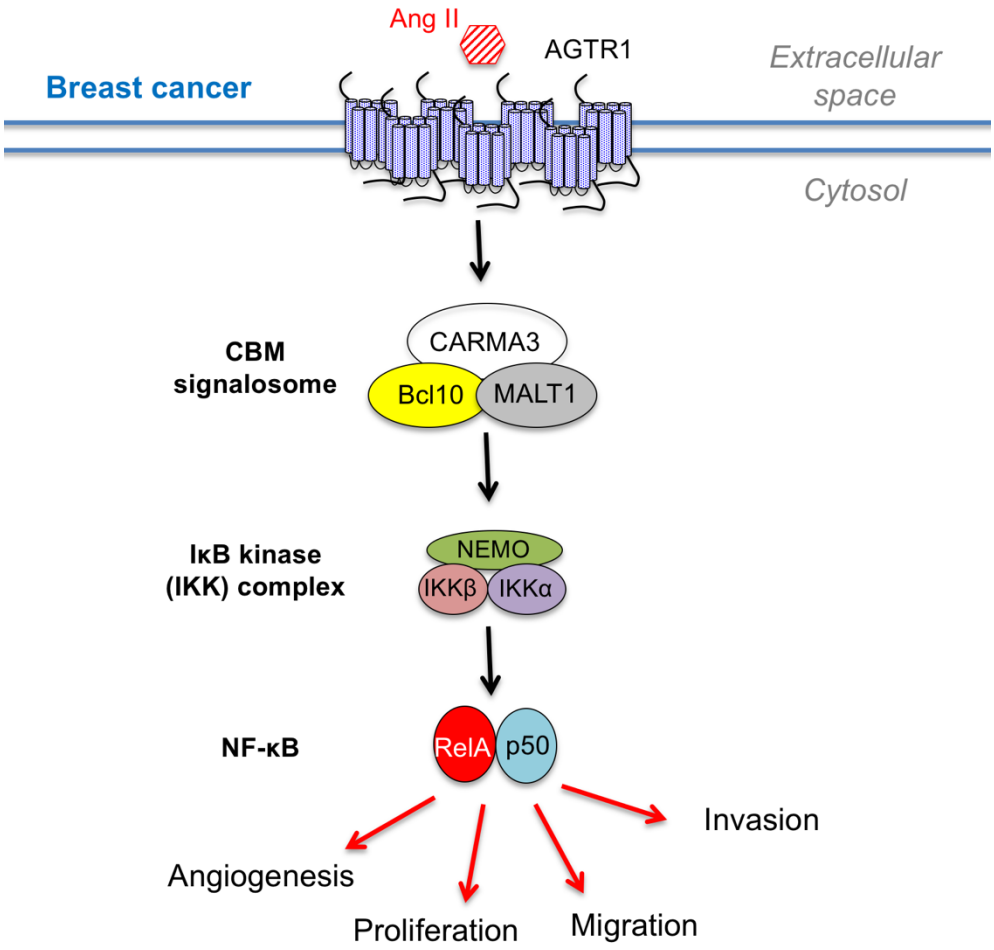


Figure 15 - The CBM signalosome promotes aggressive phenotypes in AGTR1-positive breast cancer.

Schematic summarizing the CBM signalosome-mediated NF-κB activation promotes several aggressive phenotypes (angiogenesis, proliferation, migration, invasion) in AGTR1-positive breast cancer.

3.0 Introduction – PARt 2

This section is the background introduction of my main project: epithelial-to-mesenchymal transition.

3.1 Epithelial-to-mesenchymal transition (EMT)

Epithelial-to-mesenchymal transition (EMT) is a reversible process that temporarily transforms an epithelial cell into its mesenchymal state.[99-102] During this process, epithelial cells undergo multiple biochemical changes that lead to enhanced migratory capacity, invasiveness, and elevated resistance to apoptosis. According to a proposal discussed at a 2007 EMT meeting in Poland and a subsequent meeting in March 2008 at Cold Spring Harbor Laboratories, EMT is classified into three types: the EMTs associated with implantation, embryogenesis, and organ development are categorized as type 1; the EMTs associated with wound healing, tissue regeneration and organ fibrosis are type 2; finally, the EMTs associated with cancer progression and metastasis are grouped as type 3.

3.1.1 Type 1 EMT: EMT during implantation, embryogenesis, and organ development

During the earliest stages of embryogenesis, embryo implantation and placenta formation are both associated with EMT[103]. A fertilized egg undergoes gastrulation, which starts with the formation of a primitive streak in the epiblast layer[104]. The primitive streak generates the

mesendoderm by invagination or ingression, which subsequently separates to form the mesoderm and endoderm via EMT. During embryonic development, the neural crest cells undergo EMT and then dissociate from the neural folds, display enhanced motility, and spread to different parts of the embryo for further differentiation[105,106]. These type 1 EMTs neither cause fibrosis nor induce invasive phenotype. They generate mesenchymal cells (primary mesenchyme) that might undergo MET to produce secondary epithelia.

3.1.2 Type 2: EMT associated with wound healing, tissue regeneration and organ fibrosis

During the process of wound healing, epithelial cells acquire motility via EMT and migrate across the damaged area, proliferate and then revert to the epithelial state in order to restore the integrity of the epithelial barrier[107]. However, tissue integrity needs to be restored not only through re-epithelialization but also through formation of a stress-resistant scar. The various inflammatory signals and the components of a complex extracellular matrix (ECM) released by inflammatory cells and fibroblasts will mediate organ fibrosis in epithelial tissues, while epithelial cells, via EMT, serve as important precursors of the fibroblasts that arise during this fibrosis process[108-110].

3.1.3 Type 3: EMT associated with cancer progression and metastasis

It is thought that early stage carcinoma cells are in their epithelial state, which gradually acquire mesenchymal characteristics with the progression of tumor pathogenesis. Such tumorigenesis-associated EMT results in elevated invasiveness of cancer cells and their resistance

to chemotherapy[111,112] and immunotherapy[113,114]. In addition to elevated therapeutic resistance, activating EMT process in carcinoma cells also leads to cancer stem cell (CSC) state[115,116].

3.2 Molecular and cellular changes during EMT

Normal epithelial cells display apical-basal polarity and are held together by tight junctions, adherens junctions, desmosomes, and gap junctions[117]. In general, the shape of epithelial cells is flat and polygonal. The cellular structure consists of actin cytoskeleton, a microtubule network, and intermediate filaments that support the shape of epithelial cells[118]. Induction of EMT leads to the expression of EMT transcription factors (EMT-TFs) which repress the expression of genes related to epithelial state, and activate the genes associated with mesenchymal state[119]. A hallmark of EMT is the downregulation of E-cadherin and the upregulation of N-cadherin, as known as canonical "cadherin switch", that alter cell adhesions and increase its motility[120]. Mesenchymal cells don't have uniform composition and cellular adhesion, and they possess front-rear polarity. Mesenchymal cells display irregular morphology with elongated, spindle-like shape[121]. Under this state, vimentins are upregulated to strengthen the cytoskeleton, providing more flexibility and less damage during migration[122] (Figure 16).

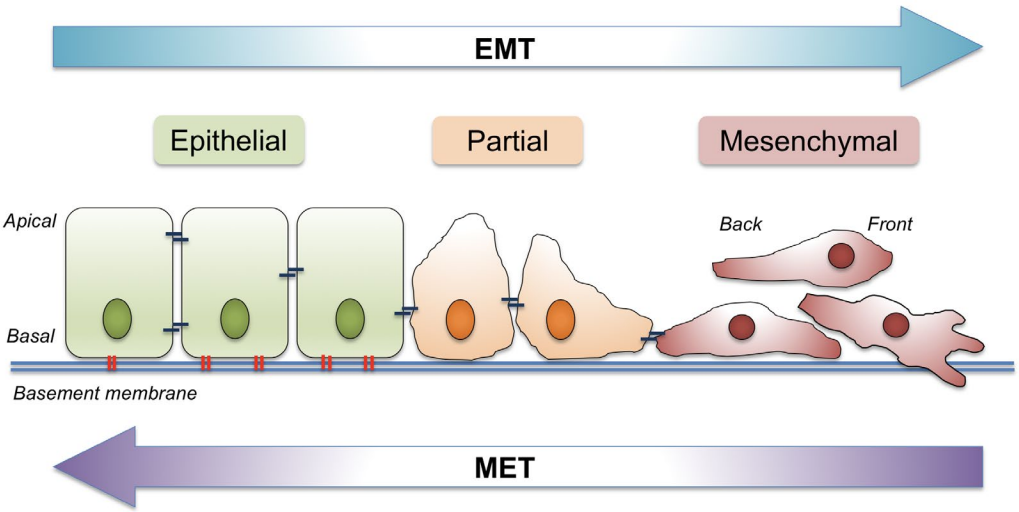


Figure 16 - Outline of typical EMT.

3.3 Transcription factors driving EMT

Numerous transcription factors contribute to the repression of epithelial genes and the activation of mesenchymal genes. These master regulators, Snail, Twist, and ZEBs, are activated during EMT and have critical roles in development, fibrosis, and tumor progression (Table 1).

3.3.1 Snail transcription factor

Among the three Snail family members, SNAI1 (Snail) and SNAI2 (Slug) activate EMT during development, fibrosis, and cancer[123]. They inhibit the expression of epithelial genes by binding to E-box DNA sequences through their carboxy-terminal zinc-finger domains[124,125]. When Snail binds to E-box sequences in the proximal promoter region of the E-cadherin gene, it recruits the Polycomb Repressive Complex 2 (PRC2), which mediates the methylation and acetylation at

histone H3, leading to the repression of E-cadherin[126,127]. They are also responsible for the inhibition of occludins and cadherin-16, while inducing the expression of mesenchymal genes (N-cadherin and ZEB1) and proteases (MMP2, MMP9)[119,127,128]. Snail and Slug can be regulated by multiple signaling pathways under different cell contexts, including the canonical TGF-β[129] or TGF-β-induced MAPK, HGF, and EGF activated ERK[130,131], GSK3β mediated Wnt[132], NF-κB[133], and Notch[134,135]. Snail collaborates with other EMT transcription factors by directly activating ZEB1 and Twist, and these transcription factors further activate Snail to stabilize and preserve the mesenchymal state[119,124,136].

The localization and degradation of Snail can be regulated by GSK3β[124]. With GSK3β-mediated phosphorylation at two Ser-rich motifs, the transcriptional activity of Snail is repressed. Phosphorylation of Ser97 and Ser101 in the first motif promotes nuclear export of Snail, while the following phosphorylation of Ser108, Ser112, and Ser120 in the second motif directs Snail toward proteasomal degradation[137]. The expression of Snail is also repressed by microRNAs miR-29b and miR-30a[138,139], therefore, increase miR-29b can reverse EMT and decrease cell invasion.

3.3.2 Basic helix-loop-helix (bHLH) transcription factors

Homodimeric and heterodimeric basic helix-loop-helix (bHLH) transcription factors are regulators of lineage specification and differentiation. Among the family of bHLH transcription factors, Twist1 and Twist2 dimerize with E12 and E47 to regulate DNA binding, inhibit epithelial gene expression, and induce mesenchymal gene expression[124,140]. In cancer cells, Twist1 inhibits E-cadherin expression and activates N-cadherin without the involvement of Snail[141,142]. Twist recruits a methyltransferase SET8 that mediates histone monomethylation, leading to the

repression of E-cadherin and activation of N-cadherin[143]. The downregulation of E-cadherin activates Twist in a feed-forward loop to maintain EMT state[144]. The expression of Twist can also be induced by hypoxia-inducible factor 1α (HIF1α) under hypoxic conditions, which promotes EMT and tumor cell dissemination[142].

3.3.3 Zinc finger E-box binding (ZEB) transcription factors

The two ZEB family members, ZEB1 and ZEB2, are zinc finger homeobox proteins and have activating and repressing functions for EMT induction[124,125]. ZEB1 has been shown to display the strongest correlation with EMT across different cancer tissues[145]. In contrast to other EMT transcription factors, ZEB1 and ZEB2 may repress the expression of epithelial genes encoding components of adherens and tight junctions, while activating mesenchymal factors such as vimentin, fibronectin, N-cadherin, and matrix metalloproteinases (MMPs)[146,147]. ZEB-mediated inhibition of E-cadherin often recruits C-terminal binding protein (CTBP) co-repressor, although in some cancer cells ZEB1 recruits the Switch/sucrose nonfermentable (SWI/SNF) chromatin remodeling protein BRG1 instead. Both ZEB1 and ZEB2 contain SMAD interacting domain (SID) that binds R-SMADs in the canonical TGF-β pathway, leading to the switch from their repressive role to activating role[148].

Since *ZEB1* gene is one of the direct targets of Snail, the expression of ZEB1 usually follows the activation of Snail. Collaborating with Snail, Twist1 can also induce the expression of ZEB1[149]. Several signaling pathways can induce the activity of ZEB1, including TGFβ, Wnt proteins, and growth factors that can activate RAS-MAPK signaling[125]. Furthermore, the expression of ZEB1 and ZEB2 is post-transcriptionally inhibited by miR-200 family and miR-

205[150]. Therefore, decreased miR-200 level during EMT leads to increased ZEB1 and ZEB2 expression and EMT progression[151].

Table 1 - EMT transcription factors and their direct targets

Transcription factor	Genes downregulated by transcription factor	Genes upregulated by transcription factor	Refs
Snail and Slug	E-cadherin, claudins, occludin, cytokeratins	Fibronectin, N-cadherin, MMPs, Twist, ZEB1, ZEB2	124,125,152 153-155
Twist1	E-cadherin, claudins, occludin	Fibronectin, N-cadherin	124,125,141 142,156
ZEB1 and ZEB2	E-cadherin, ZO1	N-cadherin, MMPs	124,125,157 148,158

3.4 EMT and the invasion – metastasis cascade

The dissemination of cancer cells from their primary sites to subsequent colonies at distal tissues require a multi-step process known as the invasion-metastasis cascade.[159-161] This process includes the invasion of primary tumors into surrounding tissues; intravasation of tumor cells into the circulatory system; spread via hematogenous dissemination to distal tissues; arrest and extravasation into distal tissues; formation of colonies and proliferation into metastatic tumor lesions.

According to recent evidence produced by different researchers, EMT might be an integral component for tumor progression of all types of carcinoma (Table 2). Ye *et al* showed that ZEB1 expression is required for efficient invasion and metastasis in a mouse model of pancreatic adenocarcinoma and that the expression of Snail is essential for the dissemination of mouse carcinoma cells[162,163]. Guo *et al* showed that expression of Slug in previously non-metastatic cells greatly elevated their metastatic ability.[164] These reports provided early proofs for the importance of EMT in the metastatic dissemination of cancer cells.

3.4.1 Cell detachment

During EMT, activated protein kinase C (PKC) pathway can inhibit the expression of claudin and occludin, leading to "leaky" and permeable tight junctions[119,165]. "Cadherin switch" occurs; E-cadherin is downregulated or degraded, further destabilizing the adherens junctions. The upregulation of N-cadherin will increase the affinity toward the surrounding mesenchymal tissues, facilitating cell migration and invasion. The overall repression of epithelial genes results in the loss of cell apical-basal polarity and intercellular attachments between cells.

3.4.2 Invasion

It has been reported that carcinoma cells from primary tumors invade adjacent tissues as clusters of cancer cells rather than as individual cells[161,163,166,167]. The expression of E-cadherin in these invading clusters might contradict what was regarded as canonical characteristics of EMT. However, some literatures suggest that cells within these clusters are undergoing partial

EMT[99,168,169]. They have reached a partially epithelial and partially mesenchymal state, in which they express a mixture of markers. Interestingly, other reports proposed that cancer cells located at the leading edge of an invading tumor display characteristics of activated EMT[162,167,170,171]. During invasion, these more-mesenchymal cancer cells at the leading front are followed by more-epithelial cancer cells located at the central area of such clusters.

3.4.3 Intravasation

Once the metastatic cancer cells have invaded into the surrounding stroma, they will disrupt the endothelial junctions in order to cross the endothelial barrier and enter the circulation. Passing through these barriers requires either active or passive migration. Active migration is facilitated by urokinase[172], cathepsins[172] and MMPs[173], cleaving cell-surface proteins and degrading the basement membrane and extracellular matrix components. Passive migration is amoeboid invasion, where cells can slide through barriers without proteolytic activity[174].

3.4.4 Arrest and extravasation

When circulating metastatic cancer cells arrive at capillaries similar to their size, they will slow down and roll along the endothelium before arresting. E-selectin expressed by metastatic cancer cells helps their migration along the endothelium. After anchorage to the endothelium, metastatic cancer cells will secrete factors that facilitate the opening of endothelial cell junctions, leading to their extravasation into the tissues of metastatic sites[175].

3.4.5 Colonization and MET

MET (mesenchymal-to-epithelial transition) is the reversed process of EMT, allowing metastatic cancer cells to regain their epithelial characteristics and the potential to colonize at distant tissue sites. How MET regulates tumor colonization still requires further investigation, however, it has been reported that many of the mechanisms involved are closely related to EMT. Several canonical EMT transcription factors are downregulated during MET, including Twist1[168], Snail and Slug[176], enabling cells to reclaim their epithelial properties and restore cell-cell contacts that facilitate colonization. Alternatively, MET can be induced via the active interference of certain uncharacterized molecules that repress EMT transcription factors[168,169,177-179]. For example, Pattabiraman *et al* had shown that extracellular signals that induced intracellular cyclic AMP (cAMP) would promote the transition of mesenchymal cells into their epithelial state[180].

Table 2 - EMT in various cancer types

Tumor type	Relevance to EMT	Refs
Breast	Snail expresses in invasive ductal carcinoma and correlates with lymph node metastasis.	181
	Twist1 promotes metastasis of mouse mammary carcinoma.	144
	HER2-induced mammary tumors express Snail and features of EMT.	182
Pancreatic	Invasive carcinoma cells display features of EMT in a mouse model of pancreatic cancer.	166
	ZEB1 severely influences cancer progression, invasion and metastasis in a mouse model of pancreatic cancer.	163
Lung	The expression level of EMT markers is tightly associated with disease progression of SCLC.	183
	EMT markers are expressed at the peripheral leading edge of NSCLCs and are correlated to cancer progression.	184
Colorectal	Slug expression is associated with tumor progression and is a negative prognostic factor for patients.	185
	N-cadherin drives cancer progression in colorectal cancer.	186
	ZEB2 correlates with tumor progression and is a prognostic marker for colorectal cancer.	187
Hepatocellular	Twist overexpression induces EMT and promotes invasion and metastasis in hepatocellular carcinoma.	188
	Snail induces EMT and promotes metastasis and tumor-initiating properties in hepatocellular carcinoma.	189

3.5 Signaling pathways that activate EMT

When epithelial cells encounter the signals released from their surrounding microenvironment, several intracellular signaling pathways will be activated, leading to the induction of EMT response. These pathways can collaborate with each other and converge on the activation of EMT.

3.5.1 Transforming growth factor-β (TGFβ)

TGFβ plays a critical role in inducing EMT in multiple tissue types[119,125,190]. TGFβ pathway is activated by ligands consisting of three distinct TGFβ isoforms, two activins, and several bone morphogenetic proteins (BMPs). TGFβ ligands bind to two type of receptors, TGFβ receptor type 1 (TGFβR1) and TGFβR2, resulting in the phosphorylation of SMAD2 and SMAD3, which then form complexes with SMAD4. These SMAD complexes will translocate into the nucleus and transcriptionally activate downstream mesenchymal genes, such as vimentin and fibronectin, as well as EMT transcription factors Snail, Slug, ZEB1 and Twist. These EMT transcription factors can further upregulate the expression of TGFβ, promoting a positive autocrine signaling loop, at the same time maintaining the expression of mesenchymal genes.

3.5.2 Wnt signaling

The canonical Wnt signaling pathway has been viewed as a key activator of EMT[191]. Various Wnt ligands can activate this pathway through binding to the Frizzled receptors, triggering

a series of molecular interactions, finally leading to nuclear translocation of β-catenin. The nuclear β-catenin can transcriptionally regulate a plethora of genes associated with cell differentiation, proliferation and tumorigenesis[119,192]. In the context of developmental EMT, canonical Wnt pathway is essential for the formation and proliferation of neural crest precursors[193]. In the context of wound healing, β-catenin activates an EMT program that promotes wound healing process[194]. Wnt signaling can also mediate EMT by activating various EMT transcription factors. With recombinant WNT3A, Wu *et al* induced the expression of Twist, Slug and N-cadherin and inhibit the expression of E-cadherin in HER2-expressing breast cancer cells [195]. Moreover, the binding of ligands to canonical Wnt receptors will induce EMT in multiple carcinoma types[192,196]. Snail directly interacts with β-catenin in the nucleus to improve its transcriptional ability[197]. In colorectal cancer cell lines, Snail overexpression elevated the expression of Wnt-regulated genes, which was abrogated by knocking down Snail with siRNA[197].

3.5.3 Notch signaling

The main role of the Notch pathway is controlling cell fate decisions, differentiation and proliferation[198-200]. Binding of Delta-like or Jagged family ligands to four isoforms of the Notch receptor (Notch1 – Notch4) will trigger a series of proteolytic cleavage events, ultimately generating an active fragment of Notch (Notch-ICD), which then translocates into the nucleus and regulates downstream gene expression. Notch pathways have been shown to be involved in EMT regulation in multiple different carcinoma types, including breast, lung, pancreatic and squamous cell carcinoma[201-203]. Notch also collaborates with TGFβ pathway to induce EMT, which is mainly mediated through the interactions with SMADs[204-206]. Moreover, Liu *et al* reported that Notch3

transcriptionally activated the expression of ZEB1 in non-small-cell lung cancer (NSCLC), while knocking down Notch3 with siRNA abolished TGFβ-induced EMT[207].

3.5.4 Mitogenic growth factors

Activating mitogenic growth factor receptors with their ligands will trigger dimerization and the subsequent autophosphorylation by receptor-associated tyrosine kinases at the intracellular domain of the receptors. This induces several downstream signaling pathways, facilitating cell growth, proliferation, as well as cell migration and invasion via the induction of EMT[208,209].

3.5.4.1 Epidermal growth factor (EGF)

After binding to the ligand EGF, epidermal growth factor receptor (EGFR) will activate EMT via the MEK-ERK signal transduction pathway, further decreasing the expression of E-cadherin[210-212]. Besides, EGF induces the Janus kinase 2 (JAK2) – signal transducer and activator of transcription 3 (STAT3) pathway, triggering EMT in multiple cancer types[213,214]. Upon EGF stimulation, STAT3 binds to the promoter of *Twist* gene in MCF7 breast cancer cell[213]. Stimulating EGFR also leads to fibroblast-like morphological changes and the upregulation of N-cadherin and vimentin in ovarian cancer cell lines[214].

It is believed that EGF synergizes with TGFβ to facilitate EMT[212]. Furthermore, the nuclear colocalization of Snail and phosphorylated SMAD2 and/or SMAD3 can be induced by EGF in breast cancer cells. Knocking down SMAD2 and SMAD3 will abrogate EGF-mediated expression of Snail, N-cadherin and vimentin[131].

3.5.4.2 Fibroblast growth factor (FGF)

By activating the MAPK and MEK-ERK pathway, several isoforms of FGF are associated with EMT induction, further activating transcription factors responsible for the EMT process[215,216]. Besides, FGF2 overexpression correlates with poor prognosis and reduced overall survival in esophageal cancer and bladder cancer[217,218]. Interestingly, analysis of The Cancer Genome Atlas (TCGA) datasets showed that FGF2 expression is positively correlated to EMT-associated genes in bladder cancer[218].

3.5.4.3 Hepatocyte growth factor (HGF)

As the ligand of MET tyrosine kinase receptor, HGF is a potent EMT activator by upregulating the expression of Snail[209,219]. HGF also triggers a morphological change toward mesenchymal features in small-cell lung cancer (SCLC) cells. Moreover, xenografts from HGF-treated cancer cells gave rise to tumors expressing Snail and fibronectin in immunocompromised host *in vovo*. Importantly, treatment with MET inhibitors could reverse this process and abrogate chemoresistance both *in vitro* and *in vivo*[220]. HGF can also enhance migratory and invasive ability of cancer cells, ultimately promoting tumor metastasis[219,221].

4.0 Results – Part 2

This section includes the results of my project "MALT1 mediates epithelial-to-mesenchymal transition in GPCR-positive breast cancer." As a follow-up study to the *Cancer Research* paper, we investigated the impact of GPCR overexpression and the role of MALT1 on epithelial-to-mesenchymal transition in breast cancer.

4.1 Overexpression of AGTR1 causes estrogen receptor alpha downregulation in breast cancer

Previously we have identified that AGTR1 overexpression occurs exclusively in a subset of estrogen receptor-positive (ER^+) tumors[10]. Recently we also demonstrated that the $AGTR1^+$ breast cancer cases were clustered within the ER^+ (luminal A and luminal B) subgroups[97]. However, we analyzed ERα and AGTR1 mRNA expression in 61 breast cancer cell lines from Cancer Cell Line Encyclopedia (CCLE) and found that 6 out of 9 AGTR1-positive breast cancer cell lines are ERα-negative (Figure 17A). Analyses from TCGA database showed that breast tumor cases with higher AGTR1 level are prone to develop recurrence and failed endocrine therapy (Figure 17B). In order to obtain appropriate tools for our breast cancer study, we established a control breast cancer cell line (ZR75-Neo) and a breast cancer cell line stably expressing AGTR1 (ZR75-AGTR1) as stated earlier[97]. Through Western blot analysis, we found that AGTR1 overexpression led to loss of ERα in ZR75-AGTR1 cell (Figure 17C), turning the ER/PR-positive

ZR75-1 cell into triple-negative breast cancer. Moreover, breast cancer cell endogenously overexpressing AGTR1 (BT549) also displays triple-negative phenotype. Although the molecular mechanism is still unclear, these findings suggest that overexpression of AGTR1, either exogenous or endogenous, causes ERα downregulation and is associated with the recurrence of breast cancer after endocrine therapy.

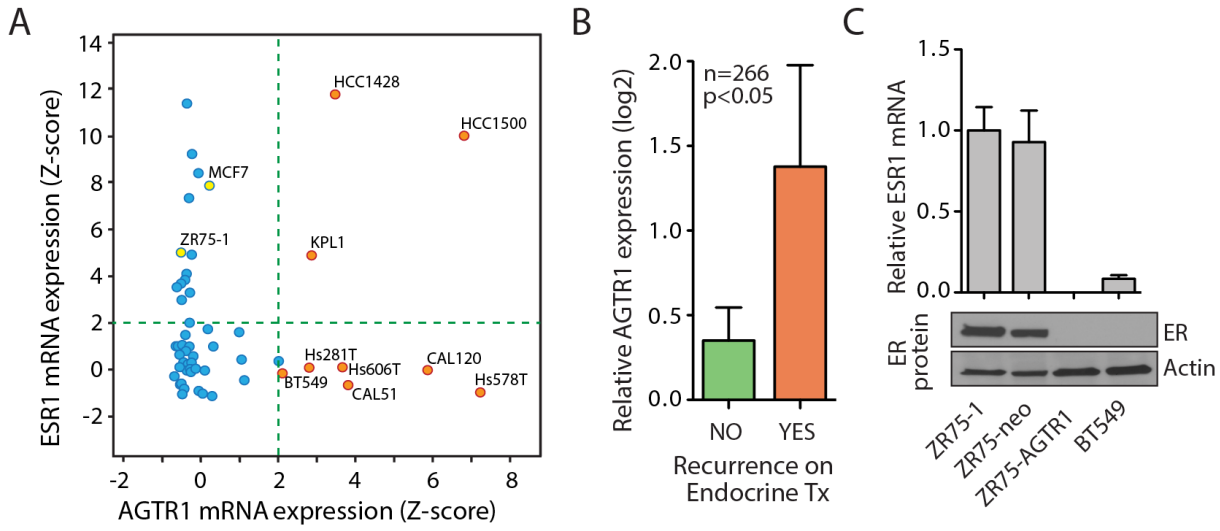


Figure 17 - AGTR1 overexpression causes ERα downregulation in breast cancer.

4.2 Overexpression of AGTR1 leads to EMT

To our surprise, overexpression of AGTR1 led to a morphological change from the rounded phenotype of ZR75-1 to the spindle-form, fibroblast-like phenotype of ZR75-AGTR1, which is consistent with the typical morphological changes when cells undergo EMT (Figure 18A). To verify this speculation, we utilized Western blot to analyze the whole cell extracts from the parental cell line (ZR75-1), the empty vector control cell line (ZR75-Neo), and cell lines overexpressing

AGTR1 exogenously (ZR75-AGTR1) or endogenously (BT549). Figure 18B shows that the AGTR1-negative breast cancer cells ZR75-1 and ZR75-Neo display strong epithelial characteristics by expressing high level of E-cadherin and lacking mesenchymal markers N-cadherin and vimentin. On the contrary, both AGTR1-positive breast cancer cells ZR75-AGTR1 and BT549 show robust level of N-cadherin and vimentin, as well as EMT factors Snail and ZEB1, which is very faint or undetectable in AGTR1-negative cells. Such observation of EMT markers in ZE75-Neo and ZR75-AGTR1 matches the canonical "cadherin switch" phenomenon during EMT, further strengthening our hypothesis that AGTR1 overexpression leads to EMT in breast cancer. Immunofluorescence staining for E-cadherin and N-cadherin in ZR75-Neo and ZR75-AGTR1 cells also demonstrated the "cadherin switch" phenomenon and correct localization of E- and N-cadherin (Figure 18C).

Since EMT has been linked to migration and invasion of cancer cells, we then looked into these aspects via Scratch wound assay and Boyden chamber invasion assay, comparing ZR75-AGTR1 cell to the parental ZR75-1 cell. As Figure 18D and E shows, AGTR1 overexpression leads to elevated migratory and invasive ability of breast cancer cell. Finally, in light of the relevance of AGTR1 overexpression and triple-negative breast cancer, we investigated the correlation of AGTR1 and EMT markers or EMT transcription factors in the triple-negative breast cancer cohort from TCGA database. Interestingly, the expression of AGTR1 is positively correlated to the expression of ZEB1 (Figure 18F) and fibronectin (Figure 18G) in triple-negative breast cancer.

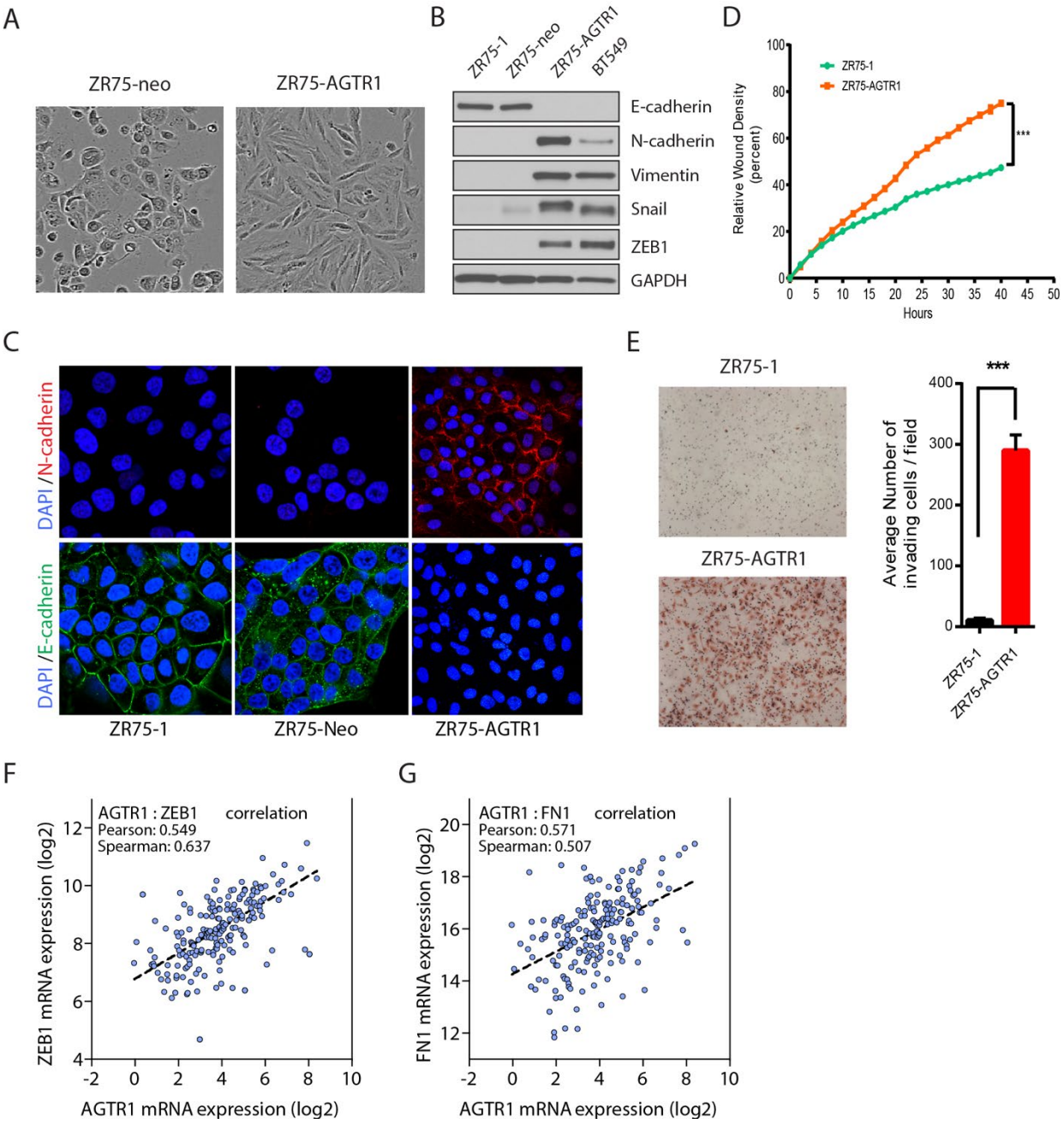


Figure 18 - AGTR1 overexpression induces EMT in breast cancer.

A. Morphological changes of ZR75-AGTR1 cells observed with IncuCyte Live Cell Imaging system. B. Immunoblots of EMT markers and transcription factors in parental and AGTR1-positive breast cancer. C. Immunofluorescence Staining of cadherins in ZR75-1, Zr75-Neo, and ZR75-AGTR1 cells. D and E, Scratch Wound healing assay and

Invasion Boyden chamber assay of ZR75-1 and ZR75-AGTR1 (***, $P < 0.001$). F and G, Correlation analysis of (F) AGTR1 vs. ZEB1 and (G) AGTR1 vs. Fibronectin.

4.3 EMT is regulated by AGTR1 and NF-κB signaling in breast cancer

To further investigate the role of AGTR1 in EMT regulation, we utilized siRNA to transiently knockdown AGTR1 in BT549 cell. Although the expression of E-cadherin was not restored after AGTR1 knockdown, the level of Snail and ZEB1 was greatly decreased, suggesting that AGTR1 overexpression might play a role in developing EMT via regulating Snail, one of the master EMT transcription factors (Figure 19A).

NF-κB signaling has long been linked to EMT in different cancer types[153,222-224]. Recently, we also demonstrated that NF-κB is one of the critical downstream components of the AGTR1/CBM complex signaling axis in breast cancer. To identify whether NF-κB regulates EMT in AGTR1-positive breast cancer, we treated BT549 cells with IKK-VI, an IKKβ-specific inhibitor, and analyzed the EMT markers and transcription factors via Western blot. The result shows that the expression of Snail is dramatically inhibited by IKK-VI (Figure 19B). Moreover, knocking down different catalytic components of IKK complex with either IKKα or IKKβ-targeting siRNAs led to the inhibition of Snail expression (Figure 19C), suggesting that NF-κB signaling is associated with EMT progression in AGTR1-positive breast cancer.

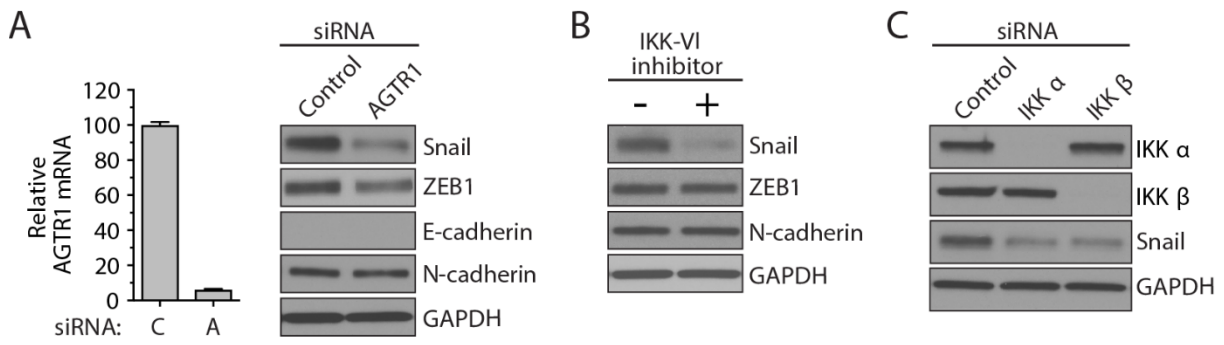


Figure 19 - AGTR1 and NF-κB regulate EMT in AGTR1-positvie breast cancer.

A, BT549 cell were transfected with non-targeting or AGTR1 siRNA. C: control siRNA; A: AGTR1 siRNA.

B, BT549 cells were treated with 5μM IKK VI for 2 days. C, BT549 cells were transfected with non-targeting, IKKα, or IKKβ siRNA. EMT markers were analyzed with Western blot.

4.4 PAR1 overexpression induces EMT in breast cancer

Our laboratory has reported that the CBM complex activates NF-κB signaling by receiving signals from different GPCRs, angiotensin II receptor type 1 (AGTR1) and protease-activated receptor 1 (PAR1), in vascular endothelial cells[49,55,98]. There are four members in the PAR family (PAR1-4). PAR1 is activated by thrombin, a serine protease produced during intravascular coagulation[225]. Thrombin cleaves the N-terminus extracellular sequence of PAR1, exposing the cryptic peptide ligand within the extracellular domain of the receptor. This tethered ligand will then bind to the residues in the conserved region of the receptor to trigger intracellular signaling[226]. PAR1 has been thought to be involved in the invasiveness and metastasis of breast[227,228], lung[229], pancreatic cancer[230], and melanoma[231-233]. Recently, people also reported that ectopic overexpression of PAR1 led to metastatic phenotype of breast cancer and regulated EMT[234].

To further investigate the role of PAR1 in breast cancer pathogenesis, we utilized an MCF7 cell line that stably overexpresses PAR1 (MCF7-PAR1) and a triple-negative breast cancer cell line that endogenously overexpresses PAR1 (MDA-MB-231). Interestingly, PAR1 overexpression leads to downregulation of ERα (Figure 20A), as was observed in AGTR1-positive breast cancer cells. Moreover, PAR1 overexpression results in EMT, which is demonstrated by downregulation of E-cadherin and upregulation of vimentin, Snail and ZEB1 (Figure 20B), as well as mesenchymal changes in morphology (Figure 20C). Transient knockdown of PAR1 in MAD-MB-231 cells leads to the reversal of EMT, demonstrated by the restoration of E-cadherin and downregulation of Snail and ZEB1 (Figure 20D). The analyses on the correlation of TCGA triple-negative breast cancer database showed that the expression of PAR1 is positively correlated to the expression of ZEB1, ZEB2 and fibronectin (Figure 20E).

Metaplastic breast cancer (MBC) is a rare form of breast cancer, accounting for 0.25-1% of breast cancer cases diagnosed annually[235]. The MBC group of tumors are morphologically diverse; different proportions or the entire tumor is composed of non-glandular epithelium or mesenchymal cells. MBCs tend to present with triple-negative ($ER^-/PR^-/HER2^-$) characteristics, and they are also considered more aggressive than triple-negative breast cancer[236]. Although MBCs originated as mammary ductal cells, they somehow undergo a change in form (metaplasia) and become completely different cells, resembling soft tissue and connective tissue in the breast. As Figure 20F shows, most MBC group of tumors overexpress PAR1 and AGTR1, which best exemplified EMT in its most dramatic fashion.

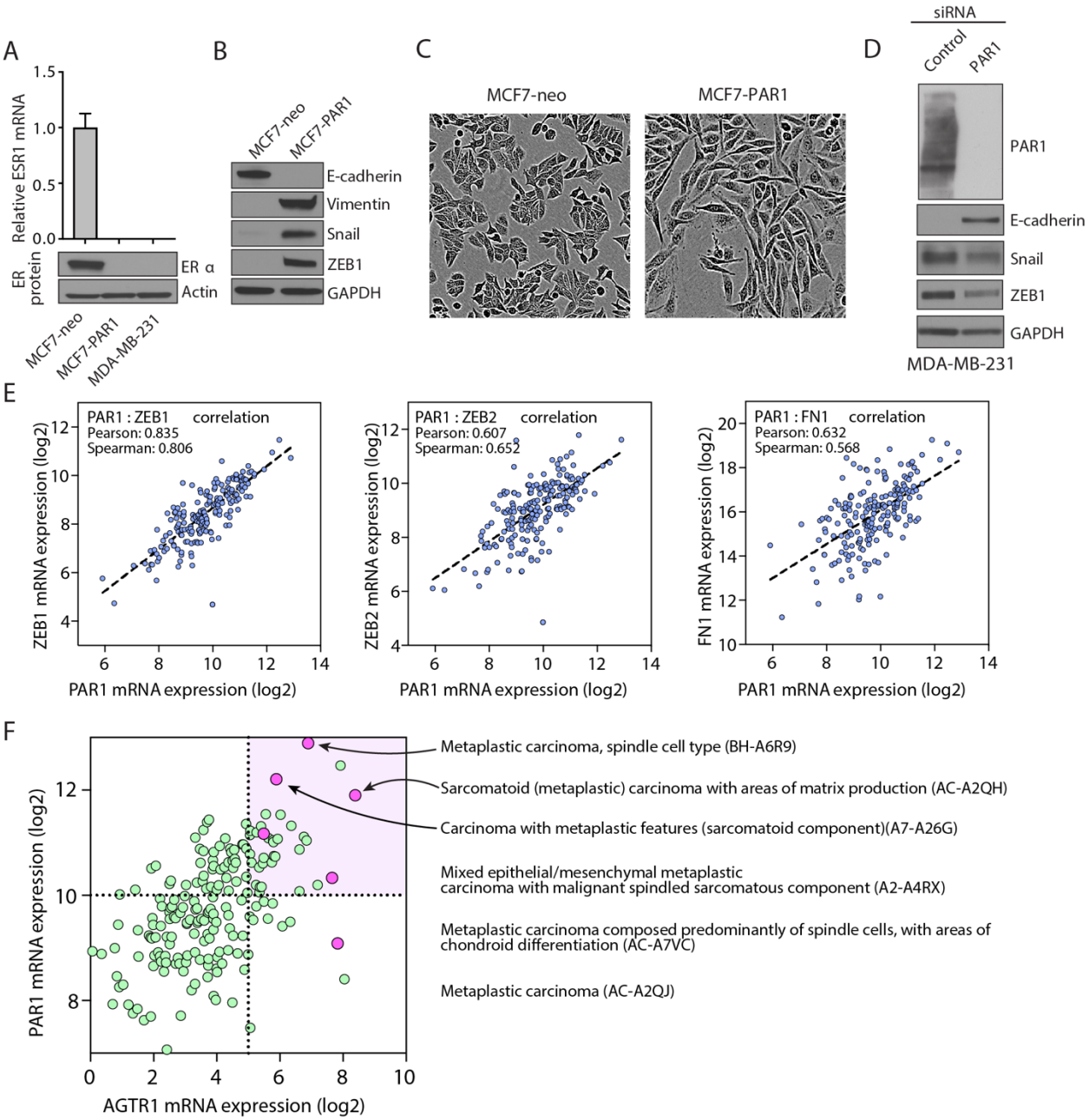


Figure 20 - PAR1 is associated with EMT in breast cancer.

A, Quantitative RT-PCR and Western blot were performed to determine the endogenous ERα mRNA and protein level in MCF7-Neo, MCF7-PAR1, and MDA-MB-231 cells. B, The EMT markers in MCF7-Neo and MCF7-PAR1 cells were detected using Western blot. C. The morphology of MCF7-Neo and MCF7-PAR1 cells was recorded by IncuCyte Live cell Imaging System. D. MDA-MB-231 cells were transfected with PAR1 siRNA. EMT markers were

analyzed by Western blot. E, Correlation analyses on PAR1 vs. ZEB1, ZEB2, and fibronectin were performed with TCGA TNBC cohorts. F, MBC group of tumors display overexpression of PAR1 and AGTR1.

4.5 GPCR-positive breast cancer is associated with EMT

Recently, Bareche *et al*[237] demonstrated that triple-negative breast cancer (TNBC) can be classified into five stable transcriptional subtypes, including basal-like 1 (BL1), basal-like 2 (BL2), immunomodulatory (IM), luminal androgen receptor (LAR), mesenchymal (M) and mesenchymal stem-like (MSL), providing potential molecular targets for TNBC therapeutics. Our laboratory has identified that overexpression of AGTR1 or PAR1 in breast cancer cells leads to downregulation of ERα, somehow turning the parental cells into their triple-negative ($ER^-/PR^-/HER^-$) status. Therefore, we investigated the dominant pathways upregulated in triple-negative breast tumor with high AGTR1 or PAR1 expression. Gene set enrichment analysis of AGTR1 (Figure 21A) and PAR1 (Figure 21B) hallmarks identifiable in triple-negative breast cancer indicated that EMT is one of the most dramatically upregulated pathways.

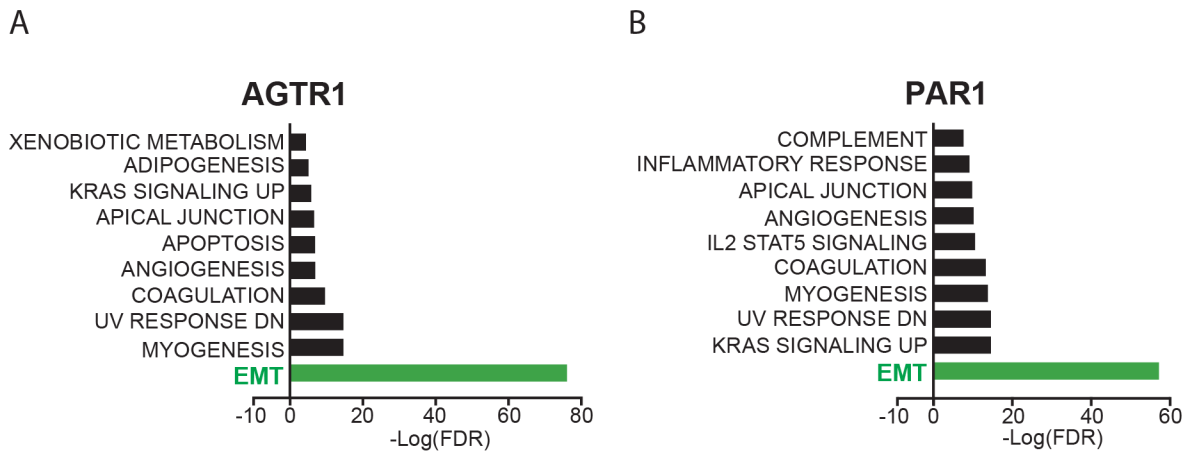


Figure 21- EMT pathway is induced in GPCR-positive breast cancer.

Key genes were analyzed using the Breast Invasive Carcinoma (TCGA, 2015 Cell) study through cBioPortal, analyzing triple-negative breast tumors. Genes set enrichment assay (GSEA) was then performed for AGTR1 (A) and PAR1 (B) in order to identify key functionality amongst these genes.

4.6 MALT1 mediates EMT in AGTR1 or PAR1-positive breast cancer

Previously our laboratory identified that the CBM signalosome is required for AGTR1-dependent NF-κB activation in endothelial cells[98] and non-immune cells[49]. Recently we demonstrated that the CBM signalosome-mediated NF-κB activation promotes the aggressive phenotypes in AGTR1-positive breast cancer[97]. Knowing that the CBM signalosome plays an important role in breast tumorigenesis, we then tried to elucidate the role of CBM complex in EMT.

Our first step was knocking down MALT1 protease with siRNA in BT549 cell. Transient MALT1 knockdown resulted in the reversal of some of the EMT markers and transcription factors, for example, N-cadherin, Snail, and ZEB1 (Figure 22A). Next, we utilized a different approach to determine the influence of MALT1 on EMT. After knocking down MALT1 in BT549 cell, total

RNAs were harvested and applied to the NanoString platform for multiplexed analysis with nCounter® PanCancer Progression Panel, which covers 770 genes from tumor progression, including angiogenesis, extracellular matrix remodeling (ECM), EMT and metastasis. The result of NanoString assay was interpreted by gene set enrichment analysis (GSEA), using the Hallmark Epithelial Mesenchymal Transition geneset[238]. As Figure 22B shows, MALT1 knockdown significantly impacted the enrichment of EMT signature genes, which were listed in the heatmap of Figure 22C.

To reconfirm the result of transient MALT1 knockdown in BT549 cell, we established Tet-on BT549 cells with inducible lentiviral MALT1 shRNAs, which inhibits MALT1 expression when treated with doxycycline. There are three different MALT1 shRNAs (#224, #226, #343-1), and all of them efficiently inhibited MALT1 expression upon doxycycline treatment, as well as leading to downregulation of Snail (Figure 22D).

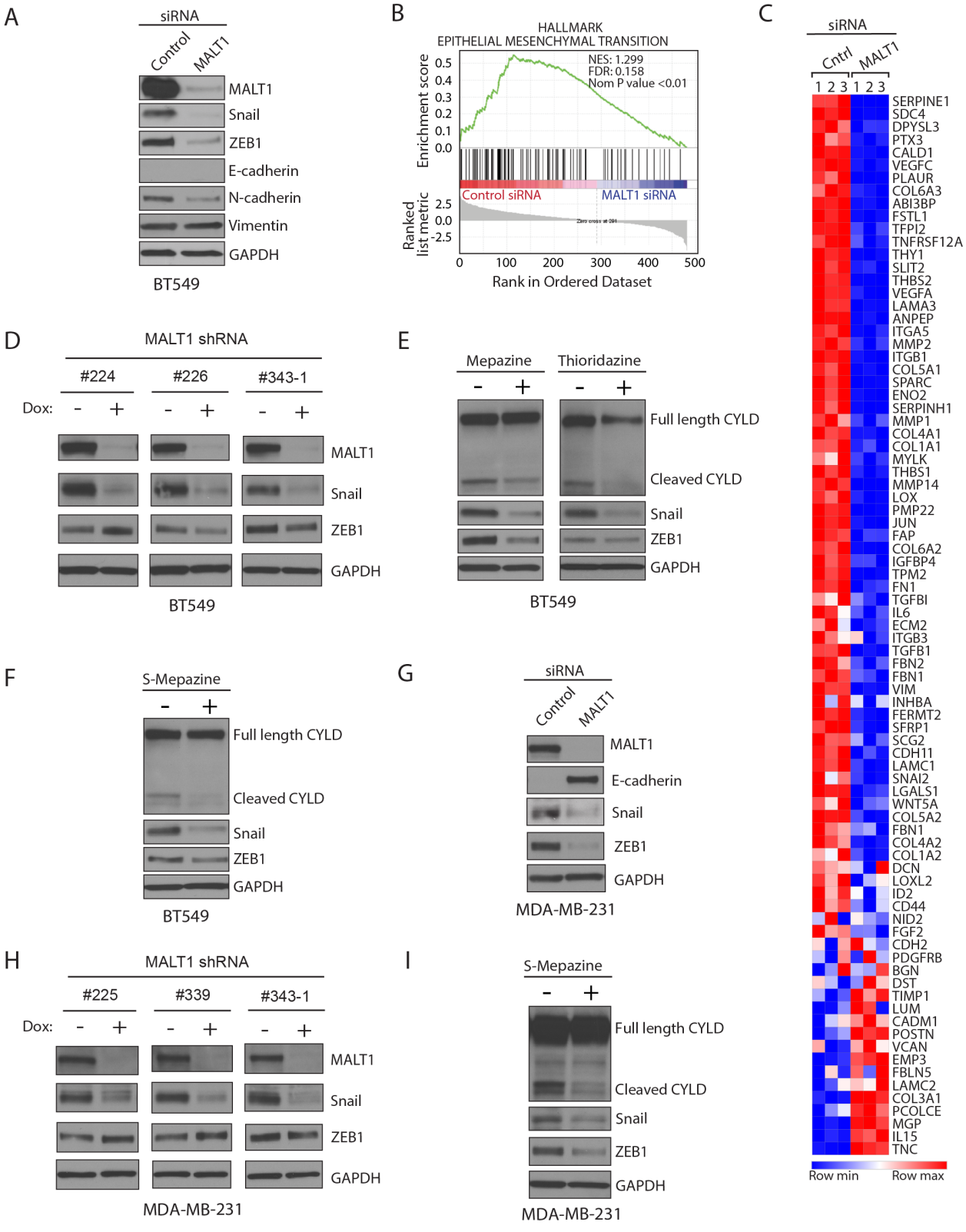

A
siRNA
Control
MALT1
MALT1
Snail
ZEB1
E-cadherin
N-cadherin
Vimentin
GAPDH
BT549
B
HALLMARK
EPITHELIAL MESENCHYMAL TRANSITION
NES: 1.299
FDR: 0.158
Nom P value <0.01
Enrichment score
Control siRNA
MALT1 siRNA
Ranked list metric
Rank in Ordered Dataset
C
siRNA
Cntrl
MALT1
1 2 3 1 2 3
SERPINE1
SDC4
DPYSL3
PTX3
CALD1
VEGFC
PLAUR
COL6A3
ABI3BP
FSTL1
TFPI2
TNFRSF12A
THY1
SLIT2
THBS2
VEGFA
LAMA3
ANPEP
ITGA5
MMP2
ITGB1
COL5A1
SPARC
ENO2
SERPINH1
MMP1
COL4A1
COL1A1
MYLK
THBS1
MMP14
LOX
PMP22
JUN
FAP
COL6A2
IGFBP4
TPM2
FN1
TGFBI
IL6
ECM2
ITGB3
TGFB1
FBN2
FBN1
VIM
INHBA
FERMT2
SFRP1
SCG2
CDH11
LAMC1
SNAI2
LGALS1
WNT5A
COL5A2
FBN1
COL4A2
COL1A2
DCN
LOXL2
ID2
CD44
NID2
FGF2
CDH2
PDGFRB
BGN
DST
TIMP1
LUM
CADM1
POSTN
VCAN
EMP3
FBLN5
LAMC2
COL3A1
PCOLCE
MGP
IL15
TNC
Row min
Row max
D
MALT1 shRNA
#224
#226
#343-1
Dox:
MALT1
Snail
ZEB1
GAPDH
BT549
E
Mepazine
Thioridazine
Full length CYLD
Cleaved CYLD
Snail
ZEB1
GAPDH
BT549
F
S-Mepazine
Full length CYLD
Cleaved CYLD
Snail
ZEB1
GAPDH
BT549
G
siRNA
Control
MALT1
MALT1
E-cadherin
Snail
ZEB1
GAPDH
MDA-MB-231
H
MALT1 shRNA
#225
#339
#343-1
Dox:
MALT1
Snail
ZEB1
GAPDH
MDA-MB-231
I
S-Mepazine
Full length CYLD
Cleaved CYLD
Snail
ZEB1
GAPDH
MDA-MB-231

Figure 22 - MALT1 mediates EMT in GPCR-positive breast cancer.

A, BT549 cells were transfected with MALT1 siRNA. B and C. The result of NanoString experiment was analyzed with gene set enrichment assay (GSEA) (B), and the heatmap of the genes from Hallmark EMT gene signature was generated (C). D, The Tet-on shMALT1-BT549 cells were treated with 2 μg/mL doxycycline for 5 days. E and F, BT549 cells were treated with 10 μM mepazine or 5 μM thioridazine for 2 days (E), or 10 μM S-mepazine for 2 days, then EMT markers were analyzed by Western blot. G, MDA-MB-231 cells were transfected with MALT1-siRNA. H, The Tet-on shMALT1 MDA-MB-231 cells were treated with 2 μg/mL doxycycline for 5 days. I, MDA-MB-231 cells were treated with 10 μM S-mepazine for 2 days, then EMT markers were analyzed by Western blot.

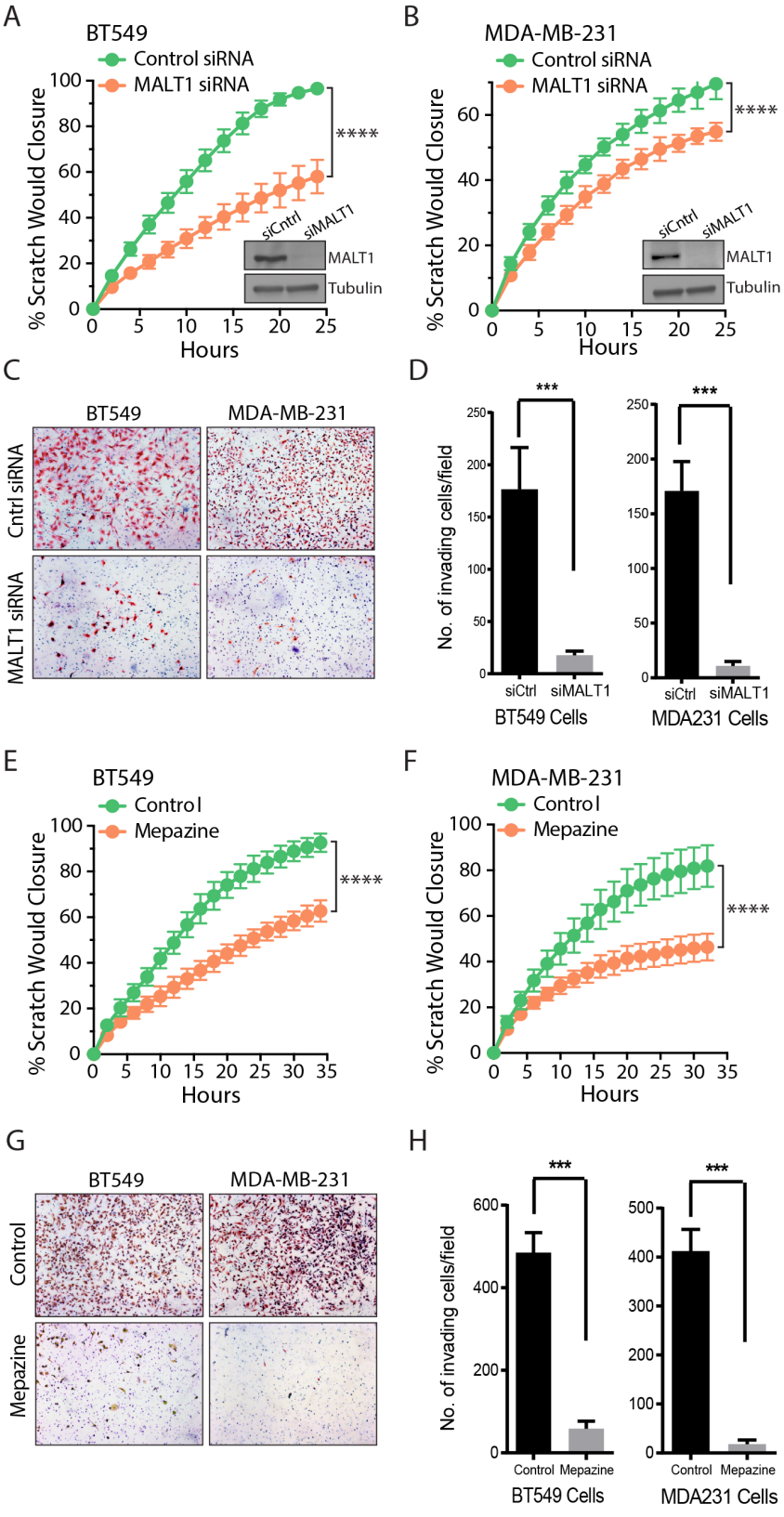
Furthermore, to determine whether the protease function of MALT1 plays an important role in EMT regulation, we treated BT549 cell with MALT1 protease inhibitor mepazine or thioridazine. Both treatments led to downregulation of Snail and ZEB1 (Figure 22E). We also treated BT549 cell with S-mepazine, an enantiomer of mepazine with significantly higher binding affinity and eight times higher inhibitory potential, and downregulation of Snail was observed after S-mepazine treatment (Figure 22F).

Knowing that the CBM complex is mediating the PAR1/NF-κB signaling, we then turned our focus to MALT1-mediated EMT in PAR1-positive breast cancer. After transiently knocking down PAR1 in MDA-MB-231 cell, we observed the reversal of several EMT markers, including the restoration of E-cadherin, and down-regulation of Snail and ZEB1 (Figure 22G). In parallel with BT549 cell, we established Tet-on MDA-MB-231 cells with inducible lentiviral MALT1 shRNAs (#225, #339, #343-1). There is a dramatic decrease in Snail level after treating these cells with doxycycline (Figure 22H). Similarly, treating MDA-MB-231 cell with S-mepazine resulted in downregulation of Snail and ZEB1 (Figure 22I).

MALT1 mediates cell migration and invasion in AGTR1 or PAR1-positive breast cancer

EMT has been linked to cell migration and invasion, so our next step is to explore the influence of MALT1 on these two biological processes associated with tumor pathogenesis. Transient knockdown of MALT1 drastically inhibited the migratory ability of BT549 cell and greatly impacted that of MDA-MB-231 cell (Figure 23A and B). The invasive ability of both BT549 and MDA-MB-231 cells was also dramatically abrogated by MALT1 knockdown (Figure 23C and D). When inhibiting MALT1 protease function in BT549 and MDA-MB-231 cells with mepazine, a dramatic decrease in migratory (Figure 23E and F) and invasive ability (Figure 23G and H) was observed, suggesting that MALT1 is important in the regulation of cell migration and invasion in AGTR1-positive or PAR1-positive breast cancers.

Recently, our laboratory collaborated with a pharmaceutical company in testing a next-generation MALT1 protease inhibitor (MALT1-IX), which displays 1000-fold higher specificity and potency in inhibiting MALT1 protease function. BT549 and MDA-MB-231 cells treated with MALT1-IX display abrogated migratory (Figure 24A and B) and invasive ability (Figure 24C and D), reconfirming that MALT1 plays an important role in GPCR-positive breast cancer pathogenesis.


A
BT549
Control siRNA
MALT1 siRNA
% Scratch Would Closure
Hours
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siCntrl
siMALT1
MALT1
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siMALT1
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Tubulin
C
BT549
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MDA-MB-231
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No. of invading cells/field
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Control
Mepazine
BT549 Cells
Control
Mepazine
MDA231 Cells

Figure 23 - MALT1 mediates cell migration and invasion in GPCR-positive breast cancer.

A and B, BT549 and MDA-MB-231 cell migration was monitored in a real-time scratch assay after transient MALT1 knockdown. C and D, BT549 and MDA-MB-231 cell invasiveness was measured with Matrigel-coated Boyden chambers after MALT1 knockdown. E and F, BT549 and MDA-MB-231 cell migration was monitored in a real-time scratch assay with 10 μM mepazine treatment. G and H, BT549 and MDA-MB-231 cell invasiveness was measured with Matrigel-coated Boyden chambers in the presence of 10 μM mepazine (***, $P < 0.001$, ****, $P < 0.0001$).

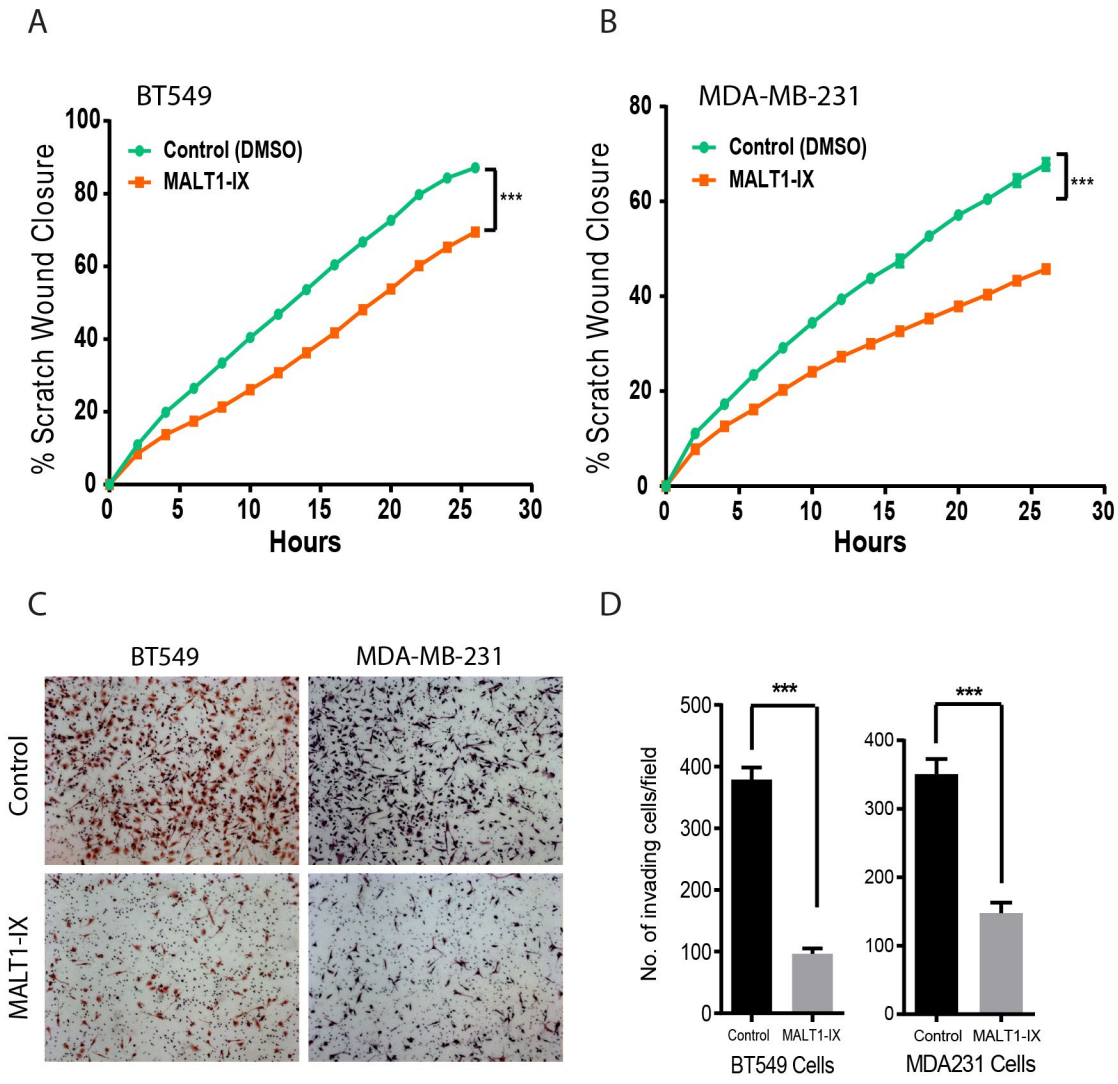

Figure 24 - Next-generation MALT1 inhibitor compound (MALT1-IX).

A and B, BT549 and MDA-MB-231 cell migration was monitored in a real-time scratch assay with the treatment of MALT1-IX. C and D, BT549 and MDA-MB-231 cell invasiveness was measured with Matrigel-coated Boyden chambers in the presence of MALT1-IX (***, $P < 0.001$).

4.7 MALT1 is associated with EMT in breast cancer

By utilizing the TCGA RNA-seq dataset of breast cancer, we selected the top 10% and bottom 10% of MALT1 expressing patients. After identifying differentially expressed genes between high and low MALT1 patients, we performed gene set enrichment analysis (GSEA) on these differentially expressed genes using Hallmark EMT gene set. Interestingly, the result showed that high MALT1 gene expression is associated with EMT (Figure 25A).

We next used the CCLE Hoeflich gene-expression dataset of 51 breast cancer cell lines to investigate the status of MALT1. After separating these cell lines into high MALT1 expression and low MALT1 expression groups, we identified 1010 significantly differentially expressed genes between these groups. We then performed GSEA on these genes using Hallmark EMT gene set, which also demonstrated a strong association between high MALT1 gene expression group and EMT (Figure 25B).

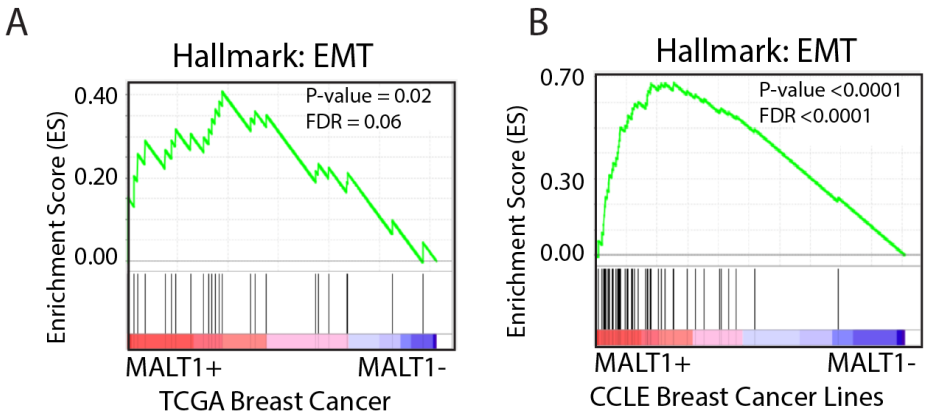

Figure 25 - MALT1 is associated with EMT in breast cancer.

Gene set enrichment assay (GSEA) was performed on TCGA breast tumor cohort (A) and CCLE breast cancer cell lines cohort (B) with Hallmark EMT signatures.

4.8 Conclusion

To sum up, we demonstrated that overexpression of AGTR1, a G-protein-coupled receptor (GPCR), leads to EMT in breast cancer. Disrupting the protease function of MALT1 with inhibitors or knocking down MALT1 with siRNAs in AGTR1-positive breast cancers all resulted in Snail downregulation, suggesting that the CBM signalosome might play an essential role in regulating EMT in breast cancer. Moreover, the overexpression of PAR1, another GPCR utilizing the same CBM signalosome pathway as AGTR1, also leads to EMT in breast cancer. Similarly, disrupting MALT1 function with inhibitors or siRNAs in PAR1-positive breast cancers resulted in the downregulation of Snail, suggesting that the CBM signalosome may have a pivotal role in regulating EMT induced by different GPCRs in breast cancer (Figure 26). Ultimately, these study provides us with a novel approach for treating GPCR-positive breast cancer with AGTR1 or PAR1 blockers and/or inhibitors of the CBM components.

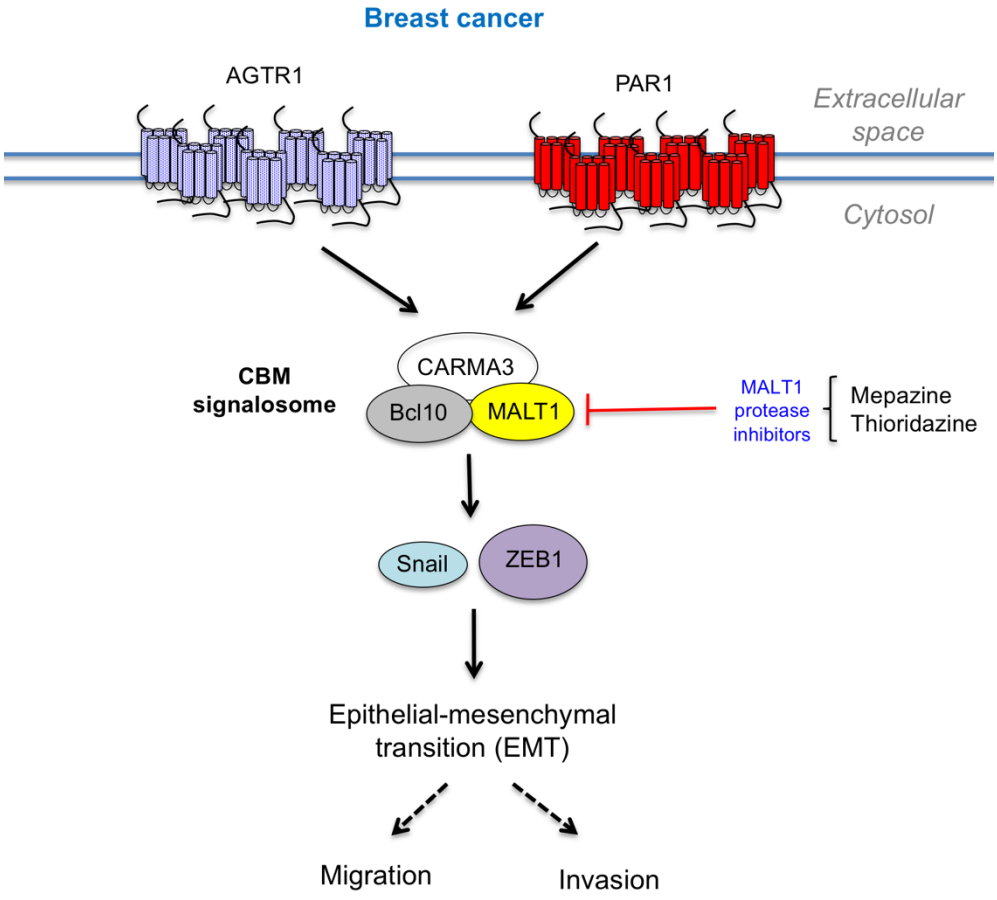

Figure 26 - The model of MALT1-mediated EMT in GPCR-positive breast cancer.

Overexpression of AGTR1 or PAR1 in breast cancer leads to the CBM signalosome-mediated epithelial-to-mesenchymal transition (EMT). Disrupting MALT1 with siRNA or MALT1 protease inhibitors reversed some of the EMT markers, especially the master EMT transcription factor Snail. MALT1 also mediates the migratory and invasive abilities, which are tightly linked to EMT, in GPCR-positive breast cancer.

5.0 Discussion

The components of CBM signalosome, CARMA1/2/3, Bcl10, and MALT1, although with different isoforms, express ubiquitously across all sorts of organs and tissues. The deregulation of the CBM signalosome has severe effects on normal biological functions, as was demonstrated in causing several types of lymphoma and carcinomas. Recently, our laboratory has shown that the CBM signalosome is mediating the angiotensin II-dependent NF-κB activation in AGTR1-positive breast cancer, which leads to the occurrence of aggressive phenotypes such as angiogenesis, proliferation, migration and invasion. Moreover, our follow-up study demonstrated that the CBM signalosome, especially MALT1, mediates EMT in AGTR1-positive breast cancer, and this concept was further expanded into breast cancers overexpressing PAR1, another GPCR that utilizes the CBM signalosome as downstream mediator. These findings not only investigated the mechanism of tumor pathogenesis, but also provided us with novel ways to treat these malignancies.

5.1 GPCR overexpression and the downregulation of Estrogen Recptor alpha

Previously, AGTR1-positive breast cancer was identified exclusively as ER^+/PR^+ breast cancer[10]. Our recent study based on PAM50 subtype analysis also clustered AGTR1-positve breast cancer cases in luminal A and B categories, consisting mainly of ER^+/PR^+ breast cancer. However, the breast cancer cell lines overexpressing endogenous AGTR1 or PAR1 in this study (BT549,

MDA-MB-231) are triple-negative breast cancer, which is different from our earlier findings in breast tumor cases. Further analyses showed that AGTR1 or PAR1 overexpression resulted in downregulation of ERα, turning the parental cells into their triple-negative status, which may provide a possible explanation for breast cancer patients having ineffective endocrine therapy. The cause of this discrepancy between GPCR-positive breast tumor and GPCR-positive breast cancer cell lines remains unclear. This might be the result of evolution or caused by the process of cell line immortalization from tumor biopsy.

5.2 Potential mechanisms of GPCRs regulating EMT

Our data shows that EMT can be induced by overexpressing AGTR1 or PAR1 in parental breast cancer cells (Figure 18 and 20). We also demonstrated that AGTR1-positive breast cancer displays endogenous NF-κB activity (Figure 8B and D) and that AGTR1 overexpression does not trigger enhanced production of angiotensin II (Figure 8G). Moreover, treating AGTR1-positive breast cancer cells with captopril, an angiotensin converting enzyme (ACE) inhibitor, or valsartan, an AGTR1 blocker, did not reverse mesenchymal markers and phenotype (data not shown), suggesting that EMT in AGTR1-positive breast cancer might be conducted through an angiotensin II-independent pathway. Interestingly, it has been reported that AGTR1 can form homodimers and heterodimers that is important for receptor functioning[239]. Such dimerization of AGTR1 with itself or with other GPCRs may contribute to endogenous NF-κB activity and lead to the development of EMT.

Furthermore, other researchers have pointed out a crosstalk between GPCRs and receptor tyrosine kinases (RTKs), with GPCR mediating the activation of membrane-associated metalloproteinases, followed by the processing and shedding of tethered RTK precursor ligands, ultimately stimulating their cognate RTKs[240-242]. Since several RTK signaling pathways have been known to promote EMT, the GPCR-mediated transactivation of RTKs may lead to the development EMT in GPCR-positive breast cancer.

5.3 EMT markers in AGTR1-positive breast cancer

Among the EMT markers and transcription factors examined, Snail was the only transcription factor that was consistently impacted by all of those siRNAs, shRNAs and MALT1 inhibitors treatments in GPCR-positive breast cancer cells. Since Snail is a direct target of NF-κB[154,243], disrupting the AGTR1/CBM/NF-κB signaling axis is likely to influence Snail expression. It has been reported that Snail is regulated by glycogen synthase kinase 3β (GSK3β)-mediated phosphorylation at two Ser residues at consensus motif, followed by nuclear exportation and proteasomal degradation[137]. However, treating BT549 cells with GSK3β inhibitor, LiCl, didn't prohibit proteasomal degradation of Snail (data not shown), suggesting that Snail might be regulated via a different mechanism in AGTR1-positive breast cancer.

The heatmap of GSEA EMT Hallmark gene set of BT549 cells after MALT1 knockdown showed the upregulation of several EMT related genes (Figure 22C). Tenascin C (TNC) is a large hexameric extracellular matrix glycoprotein that exhibits de-adhesive effects on cell-matrix interaction, enhancing cell proliferation and motility in most cell types[244]. TNC is known to be

associated with EMT, and it promotes proliferation and migratory activity of cancer cells[245-247]. TNC expression is frequently observed in invasion borders of cancer tissues where cells undergo EMT[248]. TNC is upregulated in BT549 cells after MALT1 knockdown, suggesting that it might have a compensatory role following the downregulation of mesenchymal markers and EMT transcription factors to maintain the cells in partial EMT state. This might help explain why we cannot revert GPCR-positive breast cancer cells back to their fully epithelial state by knocking down MALT1.

5.4 Developing the Malt1 protease inhibitors

MALT1 is the druggable target in the CBM complex, and several MALT1 small-molecule inhibitors have been developed and characterized, including members of the phenothiazine class (mepazine, thioridazine, promazine) and an irreversible inhibitor MI-2.[93] We have shown the effects of mepazine on the reversal of EMT markers, abrogating cell migratory and invasive abilities. However, we've been having problems utilizing MI-2 due to its cytotoxicity and narrowed window for treatment dosage. The off-target effect has always been a major concern for protease inhibitors, therefore, we need to develop a MALT1 protease inhibitor with higher specificity and milder side effect.

Recently we collaborated with a pharmaceutical company in testing a next-generation MALT1 protease inhibitor, which displays a 1000-fold higher specificity and potency in inhibiting MALT1 protease function. This next-generation MALT1 inhibitor successfully abrogated the

migratory and invasive abilities of GPCR-positive breast cancer, and we are speculating it to reverse EMT markers under optimal condition.

5.5 Potential future directions

Our study has demonstrated a novel role of the CBM signalosome-mediated tumorigenesis in AGTR1-positive breast cancer, followed by identification of MALT1 as an important EMT-mediator in GPCR-positive breast cancer. There are still some potential directions for future study:

5.5.1 Snail-mediated chemoresistance

It is well accepted that EMT plays an important role in cancer metastasis, however, EMT is also strongly associated with cancer chemoresistance[111,112,249]. Lee *et al* reported that Snail is mediating chemoresistance in colorectal cancer[250]. Since MALT1 is associated with the expression of Snail, abrogating MALT1 function with MALT1 protease inhibitors may reduce chemoresistance and benefit GPCR-positive breast cancer patients.

5.5.2 Small peptides disrupting the assembly of the CBM complex

In addition to MALT1, the other two components of the CBM complex, CARMA3 and Bcl10, can also mediate Snail expression (Figure 28). By designing small peptides to disrupt the scaffolding function of the CBM components, the aggressive phenotype of GPCR-positive breast

cancer may be further abrogated. Along with MALT1 protease inhibitors, we can develop different combinations of regimens as novel therapeutics for GPCR-positive breast cancer patients.

5.5.3 The study of MET

EMT is a highly dynamic process, with various transcription factors being expressed or inhibited at different time and space, thus generating the "partial EMT" state[102]. However, if we focus on inhibiting EMT driving transcription factors and mesenchymal markers, we might unknowingly promote the MET process happening at distant tissue sites, facilitating metastatic tumor colonization. So far people still don't know much about the MET process that promotes tissue colonization, but with the knowledge in EMT, we'll be gradually getting more in-depth understanding of MET in cancer metastasis.

5.6 Conclusion

Breast cancer is a heterogeneous disease with different molecular alterations. Our laboratory successfully identified a subpopulation of AGTR1-positive breast cancer, demonstrating the importance of the CBM signalosome in promoting the aggressive phenotypes during tumorigenesis. Introducing the role of MALT1 in mediating EMT further strengthened the multifunctional role of the CBM signalosome in cancer progression. Moreover, expanding the concept from AGTR1- to PAR1-positive breast cancer opens up the door to encompassing other membrane receptors that also utilize the CBM complex as their downstream mediator. Ultimately,

this study provides novel and alternative therapeutic options to treating GPCR-positive breast cancer, especially triple-negative breast cancer, which is in dire need of an optimal targeted-therapy.

6.0 Materials and methods

6.1 Cell lines and cell culture

BT549, ZR75-1, MDA-MB-231, and CRL-7548 cells were directly obtained from ATCC, with cell line identities confirmed by short tandem repeat (STR) profiling by the source. Frozen aliquots of cells were prepared upon receipt and all cell lines were passaged for less than 6 months. SKBR3 cells were kindly provided by Dr. Ira Bergman (Department of Pediatrics, University of Pittsburgh, PA) and the identity of this cell line was authenticated by STR profiling at the University of Arizona Genetics Core (UAGC, Tuscon, AZ). BT549, ZR75-1, and SKBR3 cells were grown in phenol red-free RPMI1640 media (catalog no: 11835030, Gibco), while MDA-MB-231 were grown in DMEM-Glutamax media, both supplanted with 10% FBS, 1% penicillin/streptomycin (Gibco), and MycoZap Prophylactic (catalog no: VZA-2032, Lonza). Lenti-Pac 293Ta cells (catalog no: CLv-PK-01) were purchased from Genecopoeia for lentiviral packaging. These cells were grown in DMEM-Glutamax media. All cells were grown at 37˚C in a 5% CO_2 incubator. Cell lines were regularly monitored for mycoplasma contamination using the mycoplasma MycoAlert Detection Kit (catalog no: LT07-318, Lonza). All cell lines were periodically authenticated by STR profiling using one of two services (ATCC or UACC).

6.2 Stable transfection and lentiviral transductions

ZR75-1 cells were transfected with either pReceiver-AGTR1-FLAG (ZR75-AGTR1) or pReceiver-FLAG (ZR75-Neo) using Lipofectamine 2000 (Thermo Fisher Scientific). After 48-72 hours, 0.4 mg/mL geneticin (G418; catalog no: 10131027, Thermo Fisher Scientific) was added to the media and cells were cultured for two weeks. Resulting G418-resistant clones were pooled and expanded further in the presence of 0.4 mg/mL G418.

Lentiviral plasmids containing either control or MALT1 shRNAs were transfected into 293Ta packaging cells using Lipofectamine 2000. Lentiviral particles were harvested, concentrated, and used to transduce BT549 and MDA-MB-231 cells for 24 hours. Selection was accomplished by culturing with puromycin and G418. Immunoblots analyses were performed to verify either the maintenance or loss of MALT1 in the resulting pools of stably transduced cells.

6.3 Transient siRNA transfection

ON-TARGET plus SMARTpool siRNAs targeting CARMA3 (catalog no: L-004395-00-0020), Bcl10 (catalog no: L-004381-00-0020), MALT1 (catalog no: L-005936-00-0020), and AGTR1 (catalog no: L-005428-00-0020) were obtained from GE Dharmacon. Nontargeting siRNA pools (catalog no: D-001810-10-50) were used as controls. SMARTpool siRNAs (20 nmol/L) were reverse transfected into BT549, ZR75-AGTR1, MDA-MB-231, or MCF7-PAR1 cells using Lipofectamine RNAiMAX (Thermo Fisher Scientific). Knockdown efficiencies were

assessed using immunoblots and real-time quantitative RT-PCR assays (TaqMan) for the intended targets after 48-72 hours.

6.4 Luciferase Reporter Assay

BT549 and ZR75-AGTR1 cells were co-transfected with an NF-κB responsive luciferase reporter (pGL4-NF-κB-luc2, Cat No: E8491, Promega) and a constitutively expressed Renilla luciferase reporter (pGL4-luc2, Cat No: E665A, Promega) in 12-well plates using Lipofectamine 3000 according to the manufacturer's instructions. 24 hours after transfection, cells were serum starved overnight and treated with Ang II (1μM) for 8 hours. When using inhibitors, cells were pre-treated with losartan (5 μM) or IKK6 (1-5 μM) for 1 hour prior to the addition of Ang II. When evaluating the impact of siRNA-mediated knockdowns, cells were first reverse transfected with siRNAs using Lipofectamine RNAiMax, and then transfected with the luciferase and renilla reporters after recovering for 24 hours. Under this scenario, Ang II treatments were formed 48-72 hours after the initial siRNA transfection. Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Cat No: E665A, Promega) according to the manufacturer's instructions. For each sample, firefly luciferase activity was normalized to that of Renilla luciferase.

6.5 SDS-PAGE, Western blotting, and quantitative RT-PCR

Cell lysates were prepared with RIPA buffer (catalog no:89901, Thermo Fisher Scientific) containing HALT protease and phosphatase inhibitor cocktail (catalog no: 78440, Thermo Fisher Scientific), loaded onto Bio-Rad 4%-15% gradient SDS-PAGETGX gels, and transferred to 0.2 μm nitrocellulose membranes (Bio-Rad). Blots were then probed with the indicated primary antibodies and developed using Pierce ECL Plus Western Blotting Substrate (catalog no: 32134). Total RNA was isolated from cell cultures and evaluated by RT-PCR using TaqMan gene expression assays (Thermo Fisher Scientific).

Table 3 – List of antibody used

Antibody	Source	Catalog No.
CARMA3	GeneTex	111222
Bcl10	Santa Cruz	Sc-9560
MALT1	CST	2494
E-cadherin	BD Biosciences	610181
N-cadherin	BD Biosciences	610920
Snail	CST	3895
ZEB1	CST	3396
Vimentin	CST	5741
PAR1	EMD Millipore	MABF244
IKKα	Santa Cruz	Sc-7218
IKKβ	Santa Cruz	Sc-8014
CYLD	Santa Cruz	Sc-74435
GAPDH	CST	5174

6.6 Immunofluorescence and confocal microscopy

ZR75-Neo and ZR75-AGTR1 cells were plated on glass-bottom 35-mm dishes (D35-20-0-N, Cellvis), 1×10^5 cells/dish. When reaching decent confluency, cells were fixed with 2% paraformaldehyde and permeabilized with 0.1% TritonX-100 in PBS. Cells were then blocked for 60 minutes and incubated overnight with mouse anti-E-cadherin (1:1000) or mouse anti-N-cadherin (1:500) antibody (BD Biosciences), followed by goat anti-rabbit (Alexa Fluor 488 or 555) secondary antibody (1:400) for 1 hour. Confocal microscopy was performed using a Zeiss LSM 710 with a 63× oil objective. Images were collected and processed using Zen software (Carl Zeiss, Inc.)

6.7 Cell proliferation, migration, and invasion assays

Two-dimensional cell proliferation was measured using the IncuCyte Live Cell Imaging System, according to the manufacturer's instructions (Essen BioScience). Migration assays were performed following the IncuCyte ZOOM 96-well Scratch Wound Cell Migration Assay protocol. Control or MALT1 siRNA-transfected BT549 and MDA-MB-231 cells were seeded at 3×10^4 cells/well in a 96-well ImageLock plate (catalog no: 4379, Essen BioScience) and incubated for 24 hours to reach confluence. A uniform scratch wound was generated in each well using the IncuCyte WoundMaker, a 96-pin mechanical device designed to create homogeneous, 700-800 micron-wide wounds in ImageLock 96-well plates. Well were then rinsed with fresh medium to remove floating cells. Remaining adherent cells were treated with either DMSO or MALT1

protease inhibitors in RPMI-1640 or DMEM containing 0.25% FBS, and the wound healing process was monitored continuously using the IncuCyte Live-cell Imaging System (Essen BioScience). Images were acquired every 3 hours over a 24-48 hour time period using a 10X objective and analyzed using the IncuCyte Cell Migration Software module.

Modified Boyden chamber invasion assay were performed using transwell chambers coated with growth factor-reduced matrigel (1 mg/mL in RPMI or DMEM SF media, catalog no: 356231 Corning). siRNA-transfected cells were seeded (1×105 cells in 0.5 mL of SF RPMI or DMEM media) into the top chamber of 24-well transwells, with or without MALT1 protease inhibitor. Medium containing 1% FBS served as chemoattractant in the bottom chamber of each transwell, in the presence or absence of MALT1 protease inhibitor. After 18 hours, residual, non-migrating cells on top surface of transwell membranes were removed, and membranes were fixed and stained according to the Diff-Stain Kit staining protocol (catalog no: K7128, IMEB Inc.). Images covering the bottom surface area of each membrane were taken with a Leica inverted microscope connected to a color camaera (4X objective). Cells in each image were counted using NIS Elements AR Analysis software (Nikon Instruments), with results from 4-5 images compiled for each membrane.

6.8 CAM Assay

The chick chorioallantoic membrane (CAM) assay was performed as a modification of a previously described method[251]. Briefly, fertilized White Leghorn eggs were purchased from a local farm and incubated at 37^{o}C and 70% humidity (G.Q.F. Manufacturing Co.). On the third day

of incubation, eggs were cracked into sterile petri dishes and incubated for 10 days. ZR75-AGTR1 cells (2 X 10^6), expressing control or Bcl10 shRNA, were re-suspended in 50 μl of RPMI SF Media containing Ang II, mixed with 50 μl of Culturex BME (18.4 mg/ml), and then pipetted slowly on to the CAM surface. ATCC-modified, HEPES-buffered RPMI SF Media was added to the surface of the CAM each day to maintain moisture and appropriate pH. After 4 days, the CAM was excised from the embryo, fixed in 10% buffered formalin, embedded on edge in paraffin blocks, and processed for routine H&E sectioning. Sections were obtained at multiple levels and reviewed in blinded fashion by a board certified Anatomic Pathologist (P.C.L).

6.9 NanoString and Ingenuity Pathway Analysis

RNA was extracted from BT549 cells 72 hours after siRNA transfection using the RNeasy Plus Kit (Qiagen) and quantified using NanoDrop, while quality was assessed using RNA Nano chips (catalog no: 5067-1511) on an Agilent 2100 Bioanalyzer. RNA samples were then evaluated using the nCounter Pan-Cancer Progression Panel (NanoString) according to the manufacturer's directions. Alternatively, samples were evaluated using a custom-designed probe panel consisting of 72 test genes and 6 housekeeping genes. Briefly, 100 ng of total RNA was hybridized overnight at 65˚C, then run on a NanoString Prep Station at maximum sensitivity. Cartridges were scanned on a NanoString Digital Analyzer at 555 fields of view. Raw count data were normalized using the nSolver analysis software version 3.0, which normalizes samples according to positive and negative control probes and the geometric mean of six housekeeping probes. Genes with

normalized counts less than 20 were considered as background and were not included in the analysis.

6.10 Bioinformatics

Publicly available gene expression data were obtained from cited studies via cBioPortal (www.cbioportal.org), the USCS Xena Browser (http://xena.ucsc.edu), and the NCBI Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo). Heatmaps were generated using the Xena Browser and Morpheus (http://software.broadinstitute.org/morpheus). GSEA analysis were performed using the GSEA software package (GSEA v2.2.3) and molecular signatures available from the Broad Institute. Clinical outcome analyses, including Kaplan-Meier plots, were performed using GraphPad Prism (v7.01) and Minitab (v17.1.0) software packages.

6.11 Statistical Analysis

Statistical analyses were performed with GraphPad Prism software. *P* values were calculated using the Student *t* test (two-sided) or through analysis of one-way ANOVA, followed by Bonferroni *post test* as appropriate. Significance was determined at $P < 0.05$.

Appendix

A.1. GPCR-POSITIVE BREAST CANCER CELL LINES SELECTION

Table 4 - GPCR-positive cell lines used in this study

This table displays different groups of GPCR-positive breast cancer cells used in our study. ZR75-1 and MCF-7 are parental cells. ZR75-AGTR1 and MCF7-PAR1 cells are generated from their parental cells and stably overexpress full-length AGTR1 or PAR1. BT549 and MDA-MB-231 cells are triple-negative breast cancer cells that endogenously overexpress AGTR1 or PAR1.

Cell lines	AGTR1	PAR1
ZR75-1	-	-
ZR75-AGTR1	Exogenous	-
BT549	Endogenous	-
MCF7	-	-
MCF7-PAR1	-	Exogenous
MDA-MB-231	-	Endogenous

A.2. INGENUITY PATHWAY ANALYSIS (IPA) OF THE NANOSTRING DATA

After the transient knockdown of MALT1 in BT549 cells, RNA was harvested and applied to the Nanostring platform as mentioned earlier. The data was analyzed with Ingenuity Pathway Analysis (IPA) software, and the result showed that EMT was the most impacted signaling pathway by MALT1 in AGTR1-positive breast cancer.

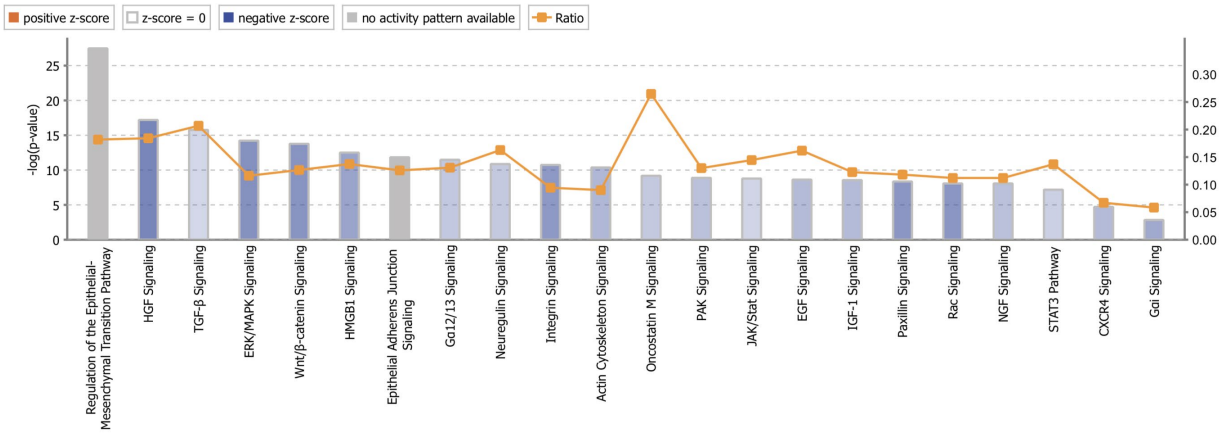

Figure 27 - MALT1 strongly impacted EMT pathway in AGTR1-positive breast cancer.

The -log (p-value) on the left Y-axis stands for the probability of a certain signaling pathway to occur. Z-score defines the directionality of pathways: Positive-Z-score means upregulation of a pathway, while negative-Z-score represents downregulation of a pathway. The ratio value on the right Y-axis stands for the percentage of genes among total genes of a certain pathway that are detected in this assay.

A.3. THE CBM SIGNALOSOME MEDIATES EMT IN BT549 CELL

Transient knockdown of each component of the CBM signalosome resulted in the downregulation of Snail, while CARMA3 and MALT1 knockdown lead to downregulation of ZEB1.

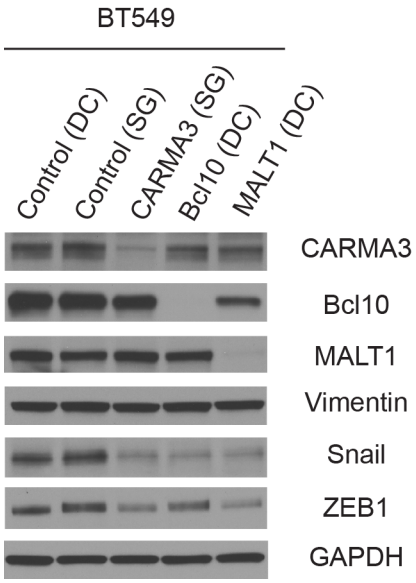

Figure 28 - The CBM signalosome mediates EMT in BT549 cell.

BT549 cell was transfected with siRNAs targeting each component of the CBM complex. DC: siRNA purchased from Dharmacon. SG: siRNA purchased from Sigma.

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